

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 17.May.02		3. REPORT TYPE AND DATES COVERED THESIS
4. TITLE AND SUBTITLE ROLE OF MEXL IN PSEUDOMONAS AERUINOSA MEXJK EFFLUX OPERON EXPRESSION			5. FUNDING NUMBERS	
6. AUTHOR(S) CAPT NARASAKI CRAIG T				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) COLORADO STATE UNIVERSITY			8. PERFORMING ORGANIZATION REPORT NUMBER CI02-70	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) THE DEPARTMENT OF THE AIR FORCE AFIT/CIA, BLDG 125 2950 P STREET WPAFB OH 45433			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION AVAILABILITY STATEMENT Unlimited distribution In Accordance With AFI 35-205/AFIT Sup 1			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)				
20020523 159				
14. SUBJECT TERMS			15. NUMBER OF PAGES 91	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT	

THESIS

ROLE OF MEXL IN *PSEUDOMONAS AERUGINOSA* MEXJK EFFLUX OPERON
EXPRESSION

Submitted by

Craig T. Narasaki

Department of Microbiology

In partial fulfillment of the requirements

for the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2001

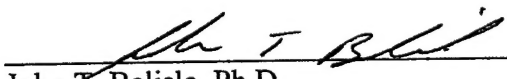
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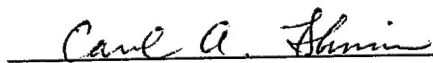
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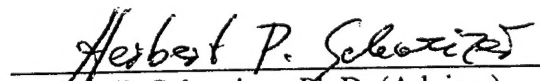
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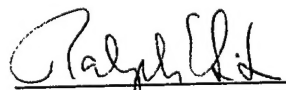
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ABSTRACT

ROLE OF MEXL IN *PSEUDOMONAS AERUGINOSA* *mexJK* EFFLUX OPERON EXPRESSION

The purpose of this study was to determine the regulatory mechanism(s) of a hitherto uncharacterized resistance nodulation cell division (RND) efflux pump, MexJK, in *Pseudomonas aeruginosa*. The clinical significance of this efflux pump has not been determined. However, it is widely accepted that RND efflux pumps in conjunction with *P. aeruginosa*'s low permeability barrier significantly contribute to its intrinsic resistance to antibiotics, and this bacterium is therefore considered one of the most important nosocomial disease causing agents of our time.

A gene encoding a protein belonging to the tetracycline repressor family of proteins (PA3678) and designated *mexL* is divergently transcribed from and located 94 bp upstream of the *mexJK* operon. It is my contention that MexL represses the MexJK efflux pump by binding via its putative helix-turn-helix motif on or before the promoter-containing region of the *mexJK* operon.

A chromosomal Δ *mexL* mutant named PAO318 was engineered from a *mexAB-oprM* and *mexCD-oprJ* double knockout mutant, PAO238. Phenotypic characterization of PAO318 by minimum inhibitory concentration (MIC) tests, revealed that PAO318 was

resistant to triclosan at >128 µg/ml. Furthermore, complementation of PAO318 with a plasmid born *mexL* reduced the MIC for triclosan to 20 µg/ml, which is consistent with the MIC of the susceptible parent strain, PAO238.

To quantitate transcriptional repression by MexL, *lacZ* operon fusions were constructed with the *mexJK* operon promoter. The β-Galactosidase activity observed in these strains suggest that MexL represses the expression of *mexJK* by at least 4 fold. To examine the possibilities of auxiliary regulatory mechanisms on this efflux pump, these *lacZ* fusions were introduced into *Escherichia coli* by λ phage transduction. Although the repressional activity of MexL in *E. coli* is consistent with that observed by fusion analysis in *P. aeruginosa*, the overall observed β-Galactosidase activities were significantly lower. This suggests that an activator of the *mexJK* operon may be present in *P. aeruginosa* that is absent in *E. coli*. Alternatively, the *mexJK* operon promoter may not be as active in *E. coli*.

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ACKNOWLEDGEMENTS

I would like to acknowledge the Biology Department at the United States Air Force Academy for helping to fund my research at Colorado State University. Serving the Air Force in this capacity has been a privileged honor.

I am very grateful to the Department of Microbiology for admitting me into the graduate program under the Department of Defense's rigid timing requirement for graduation. In this regard, I would like to extend my gratitude to Dr. Ralph E. Smith who allowed me to forgo the customary graduate student laboratory rotations, which allowed me to focus immediately on my laboratory research. Warmest thanks to Dr. John T. Belisle and Dr. Carol A. Ishimaru for serving on my graduate committee.

My deepest gratitude and debt goes to Dr. Herbert P. Schweizer for providing me with considerable guidance and inspiration throughout my first exposure to scientific research. Dr. Schweizer was patient and at the same time demanding. He acted like a protective father in many situations, but was not afraid to let me "sink or swim" on my own! He is the kind of scientist and mentor I will continually strive to be.

Nazir Barekzi, John Cusak, RoxAnn Karkhoff-Schweizer, and Sarah Sullivan have always been the best of friends who were never too busy to help. Finally, Rungtip "Jeed" Chuanchuen has been an absolutely excellent mentor to me. She is a trusted friend who I will never forget.

DEDICATION

I dedicate this work to my loving wife of nearly ten years and to my three children. Without their support none of this would have been possible.

I would also like to dedicate this work to my mom. She has always given me the encouragement to do my best.

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Explanation</u>
$A_{420\text{nm}}$	absorbance at 420 nm
$A_{540\text{nm}}$	absorbance at 540 nm
$A_{600\text{nm}}$	absorbance at 600 nm
ABC	ATP binding cassette
Acr	acriflavine
ADPRT	adenosine diphosphate-ribosylating toxin
AI	autoinducer, acyl homoserine lactone
AIDS	acquired immune deficiency syndrome
Am	amoxicillin
Ap	ampicillin
β -Gal	beta galactosidase
Bis	dichlorophene
<i>bla</i>	β -lactamase encoding gene
bp	base pair(s)
$^{\circ}\text{C}$	degrees Celsius
CAA	casamino acids
Cb	carbenicillin

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CFU	colony forming unit(s)
Cip	ciprofloxin
Cm	chloramphenicol
Cp	ciprofloxacin
Ct	cephalothin
CTX	cytotoxic
Δ	delta
<i>dam</i>	DNA deoxyadenosine methylase
dH ₂ O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate(s)
<i>E.</i>	<i>Escherichia</i>
EDTA	ethylenediamine tetraacetic acid
Ery	erythromycin
FAB	fatty acid biosynthesis
Fc	fragment crystallizable, non-antigen binding portion of an antibody
<i>FRT</i>	Flp recombinase target
Fus	fusidic acid
g	gram(s)

genome	chromosomal and plasmid DNA
Gm	gentamycin
h	hour(s)
Hch	hexachlorophene
HSL	homoserine lactone(s), autoinducer
IM	inner membrane
<i>int</i>	integrase encoding gene
kb	kilobase(s)
kDa	kilodalton(s)
Km	kanamycin
kV	kilovolt
λ	lambda, microliter
l	liter
LB	Luria-Bertani
LPS	lipopolysaccharide
M	molar
M9	minimal media
Mb	megabase(s)
MCS	multiple cloning site
MDR	multidrug resistant
Mex	multidrug efflux
MexA	periplasmic component of MexAB-OprM
MexB	inner membrane component of MexAB-OprM

MexC	periplasmic component of MexCD-OprJ
MexD	inner membrane component of MexCD-OprJ
MexE	periplasmic component of MexEF-OprN
MexF	inner membrane component of MexEF-OprN
MexJ	periplasmic component of MexJK
MexK	inner membrane component of MexJK
MexL	negative regulator of <i>mexJK</i>
MexR	negative regulator of <i>mexAB-oprM</i>
MexS	negative regulator of <i>mexT</i>
MexT	positive regulator of <i>mexEF-oprN</i>
MexX	periplasmic component of MexXY
MexY	inner membrane component of MexXY
MexZ	negative regulator of MexXY
MFS	major facilitator superfamily
MFP	membrane fusion lipoprotein
mg	milligram(s)
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
min	minute(s)
ml	milliliter(s)
mM	millimolar(s)
<i>nfx</i>	norfloxacin resistance determinate
NfxB	negative regulator of <i>mexCD-oprJ</i>

ng	nanogram(s)
nm	nanometer(s)
nM	nanomolar(s)
Ω	Ohm(s), electrical resistance
OD	optical density
OM	outer membrane
<i>omp</i>	outer membrane protein encoding gene
O.N.	overnight
ONPG	ortho-nitro-phenol- β -D-galactopyranoside
OprJ	outer membrane component of MexCD-OprJ
OprM	outer membrane component of MexAB-OprM
OprN	outer membrane component of MexEF-OprN
<i>ori</i>	gene that codes for <i>E. coli</i> origin of replication
<i>ori</i> ₁₆₀₀	pRO1600 broad-host-range origin of replication
<i>oriT</i>	origin of transfer
<i>P.</i>	<i>Pseudomonas</i>
p	plasmid
PAO	acronym for <i>Pseudomonas aeruginosa</i>
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIA	<i>Pseudomonas</i> isolation agar
<i>PmexJ</i>	promoter of the <i>mexJK</i> operon
<i>PmexJK</i>	promoter of the <i>mexJK</i> operon

PMF	proton motive force
PMNs	polymorphonuclear cells
pmol	picomole(s)
Pol	polymerase
PQS	<i>Pseudomonas</i> quinolone signal
psi	pounds per square inch
r	resistant/resistance
<i>rec</i>	recombinase encoding gene
<i>rep</i>	gene that codes for replicase required for replication from <i>ori</i> ₁₆₀₀
RND	resistance-nodulation-cell division
rpm	rounds per minute
RT	room temperature
s	second(s)
<i>sacB</i>	sucrase encoding gene from <i>Bacillus subtilis</i>
SDS	sodium dodecyl sulfate
SMR	small multidrug resistance family
Std	standard(s)
T4	type, fourth of seven bacteriophages
TAE	tris-acetate-EDTA
<i>Taq</i>	<i>Thermus aquaticus</i> DNA polymerase
TB	tryptone broth
TBE	tris-borate-EDTA

Tc	tetracycline
Tmp	trimethoprim
TMS	transmembrane segment
Tri	triclosan
μF	microfarad(s), capacitance
μg	microgram(s)
μl	microliter(s)
μM	micromolar(s)
μmol	micromole(s)
U	unit(s)
V	volt(s)
VBMM	Vogel and Bonne minimal medium
wt	wild-type
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

CHAPTER 1

INTRODUCTION

1.1 MULTIDRUG RESISTANCE

During the pre-antibiotic age, bacteremia, endocarditis, mastoiditis, meningitis, pneumonia, rheumatic fever, syphilis, tuberculosis, plague, and typhoid fever shaped human history. Infectious diseases have caused genocide, claimed the lives of our brightest leaders, and influenced the outcome of military campaigns through out history (72). In many third world nations infectious disease continues to be the number one cause of morbidity and mortality.

By the mid 1930's antibiotics were developed and saved millions of people from certain agony and/or death. The discovery of antibiotics is clearly a milestone in human evolution. It is a testament to our ability to understand and manipulate the environment in a way that is favorable to our advancement. In 1979 John Whaptesna, Surgeon General of the United States, will be remembered in infamy when he proclaimed, "We can now close the book on infectious diseases. This office believes that all bacteria borne infections have now been conquered" (72). Not long after this statement was made, it became clear that the scientific and medical communities grossly underestimated the resilience of pathogenic microbial organisms. Wide spread misuse of antibiotics have

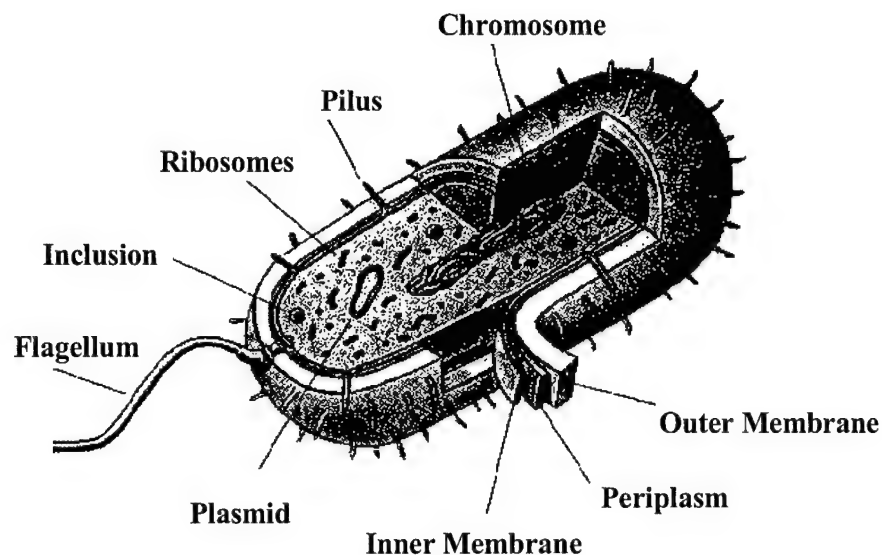
resulted in deadly multidrug resistant strains of bacteria, and has hurled us into what many believe to be the “Post-Antibiotic Era” (13).

According to Julian Davies of the University of British Columbia, Canada, scientists in the 1940’s considered the possibility of bacterial resistance. Unfortunately, however, they failed to recognize the many different mechanisms bacteria use to achieve resistance. Scientists of the day generally considered antibiotic-target-site mutation as the main mechanism of resistance. The low mutation rate of most bacteria, and the belief that genetic transfer between bacterial species were impossible, led them to believe that wide spread antimicrobial resistance would be unlikely (14).

Bacteria are the most physiologically diverse organisms on Earth (70). During their billions of years of evolution bacteria have developed “self protection mechanisms” via antibiotics to compete against neighboring bacteria (22). Through natural selection they obtained *de novo* or intrinsic resistance mechanisms to cope with the antibiotics they produce and secrete. For example, *Streptomyces griseus* produces an aminoglycoside phosphotransferase and is resistant to streptomycin (22). Bacteria were also selected for the ability to acquire resistance via transformation, transduction, conjugation, transposons, and integrons (14, 22, 49, 70). It stands to reason, that bacteria such as *Pseudomonas aeruginosa* have an entire armamentarium of resistance mechanisms (figure 1.1).

Intrinsically resistant bacteria are naturally resistant. For example, it may lack the antimicrobial target site, the antibiotic may not be able to penetrate the cell envelope, or the microbe may have chromosomally encoded DNA which codes for enzymes that can inactivate an antibiotic. On the other hand, bacteria that have acquired resistance were

A.



B.

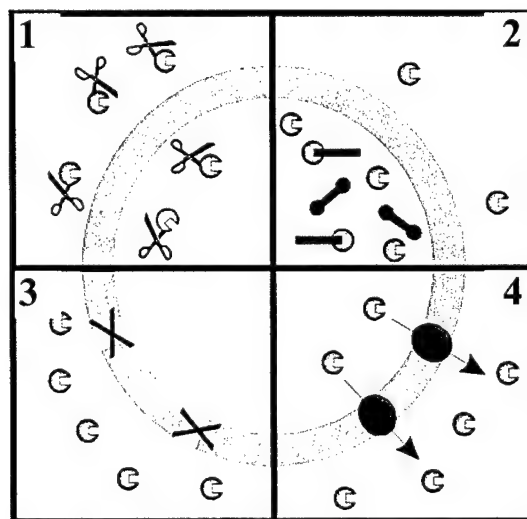


Figure 1.1. *P. aeruginosa* and Its Resistance Mechanisms. **A.** *P. aeruginosa* is a gram negative, aerobic, motile, rod shaped bacterium approximately 1 μm wide and 5 μm long with a polar flagellum. Its genome is 6.3 Mb in size and has been sequenced. **B.** General Resistance Mechanisms of bacteria. [1] drug inactivation, [2] target alteration, [3] prevention of drug influx, and [4] active extrusion of drug from the cell (9, 57, 69) .

once sensitive to a particular antibiotic. Acquired genes that confer resistance may modify the antibiotic, allow the bacterium to utilize an alternate metabolic pathway, or efflux the antibiotic out of the cell. Spontaneous mutations may also cause resistance by changing an antimicrobial target site, and decreasing antimicrobial transport into the cytoplasm of the cell (22, 72).

In 1992 Dr. Cohen of the National Centers for Infectious Disease warned of the rapidly rising number of multidrug resistant bacteria, but stated “many bacteria remain fully susceptible to commonly used antimicrobial agents.” Today “there are no antimicrobial agents used in human medicine for which resistance has not been recognized” (figure 1.2) (72). Gram positive bacteria have reached their “greatest prominence” in the past 18 years achieving universal resistance to chloramphenicol and tetracyclines (47) .

Bacterial drug resistance is caused by inappropriate use of existing antimicrobials, which allows for the natural selection and proliferation of drug resistant strains (49). Increasing human population and life expectancy, resulting in an increased number of susceptible hosts, exacerbates this problem. Many are calling for worldwide laws, citing the unabated spread of bacterial diseases while the human response is limited by bureaucracy (18). In the mean time, the scientific community continues to elucidate the mechanisms of antibiotic resistance in hopes of discovering broad spectrum “magic bullets” that will ideally be impossible to circumvent.

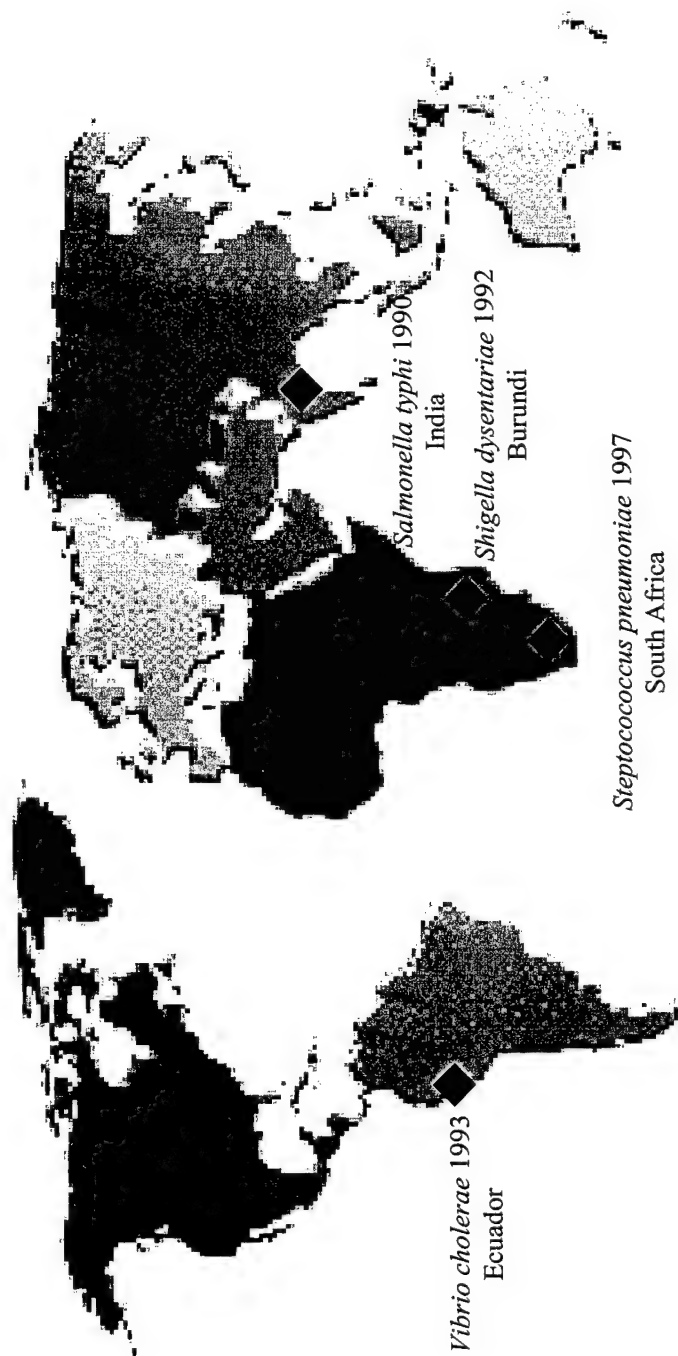


Figure 1.2. World Map Showing Several Examples of the Prevalence of Certain MDR Bacteria (modified from 72).

1.2 MULTIDRUG EFFLUX PUMPS

Researchers initially believed that antibiotic-target-site mutation was the primary mechanism of microbial drug resistance. It was later discovered that the low permeability of hydrophobic antibiotics such as β -lactams, tetracyclines, chloramphenicol, and fluoroquinolones in gram negative bacteria plays an essential role in microbial resistance (36, 38, 51).

Only recently has the importance of drug efflux been elucidated. The first studies of efflux pumps focused on cation systems, which expelled Na^+ , K^+ , and Ca^{2+} (36). In 1980 the tetracycline efflux pump of *E. coli* was discovered by McMurry et al. (44). While this pump is specific for tetracycline, other translocators were discovered that have a wide substrate capacity. These translocators are called multidrug efflux (Mex) pumps and are the subject of intensive research (36, 52). Multidrug translocators are of particular concern because of their efficacy in expelling seemingly divergent compounds. Since most antimicrobials are designed as broad spectrum agents to reduce manufacturing costs, bacteria with these translocator systems often times have little trouble becoming drug resistant (36, 52, 57).

Underscoring the importance of efflux pumps is the fact that they are found in a wide range of organisms including humans, fungi, protozoan, and bacteria (36) (table 1.1). Perhaps this is true because the role of such transporters is to expel a wide range of compounds, not just antibiotics.

Convincing data exist that suggest efflux pumps evolved fortuitously from simple transporters (37, 50). Others argue that they evolved specifically to expel toxic compounds (37, 50).

Table 1.1
Organisms and Selected Efflux Pumps^a

Organism	Efflux Pump	Efflux Pump Family
<i>Homo sapiens</i>	P-glycoprotein ^b	ATP Binding Cassette
<i>Staphylococcus aureus</i>	QacA	Major Facilitator Superfamily
<i>Escherichia coli</i>	TetB ^c	Major Facilitator Superfamily
<i>Escherichia coli</i>	EmrE	Small Multidrug Resistance Family
<i>Staphylococcus aureus</i>	Smr	Small Multidrug Resistance Family
<i>Pseudomonas aeruginosa</i>	MexAB-OprM	Resistance Nodulation Cell Division Family
<i>Neisseria gonorrhoeae</i>	MtrD	Resistance Nodulation Cell Division Family

^a Compiled from (36, 44)

^b P-glycoprotein is the best characterized eukaryotic efflux pump. It effluxes anti-tumor chemotherapeutic agents.

^c The TetB efflux pump was the first prokaryotic efflux pump discovered in 1980 by McMurphy et al. (44).

Regardless of their origins, they are an effective resistance mechanism against medically important antibiotics, and are therefore viable targets for drug discovery.

Transporters are divided into two major groups: (1) Primary transporters which are powered by an energy producing metabolic event, for example hydrolysis of ATP in ABC transporters, and (2) secondary transporters which are powered by electrochemical gradients such as the proton motive force (PMF) (76).

The PMF is an electrochemical gradient that is generated when protons are translocated across a membrane by respiration, photosynthesis, or ATP hydrolysis. The imbalance of protons creates a net positive charge on the outside of the cell and a net negative charge on the inside of the cell. Its power is harnessed when the protons return to the inside of the cell through coupling sites (power generators), which for example are the inner membrane components of efflux pumps (76). Most of the clinically significant efflux pumps in bacteria belong to the secondary transporters and are further divided into several superfamilies, the Major Facilitator Superfamily (MFS), Small Multidrug Resistance (SMR) family, and the Resistance Nodulation Cell Division (RND) family (52).

Of these superfamilies, RND efflux pumps have the broadest substrate specificity. Like other transporters, the inner membrane proteins of RND transporters have multiple transmembrane segments (TMS) (figure 1.3). In gram negative bacteria, RND transporters usually interact with two auxiliary protein families, membrane fusion lipoproteins (MFP) and outer membrane proteins (OMP), to successfully efflux their substrates, (52) (figure 1.4). *P. aeruginosa* has twelve RND-type efflux pumps, four of which have been characterized in detail (1, 33, 54, 55, 69, 75) (figure 1.5).

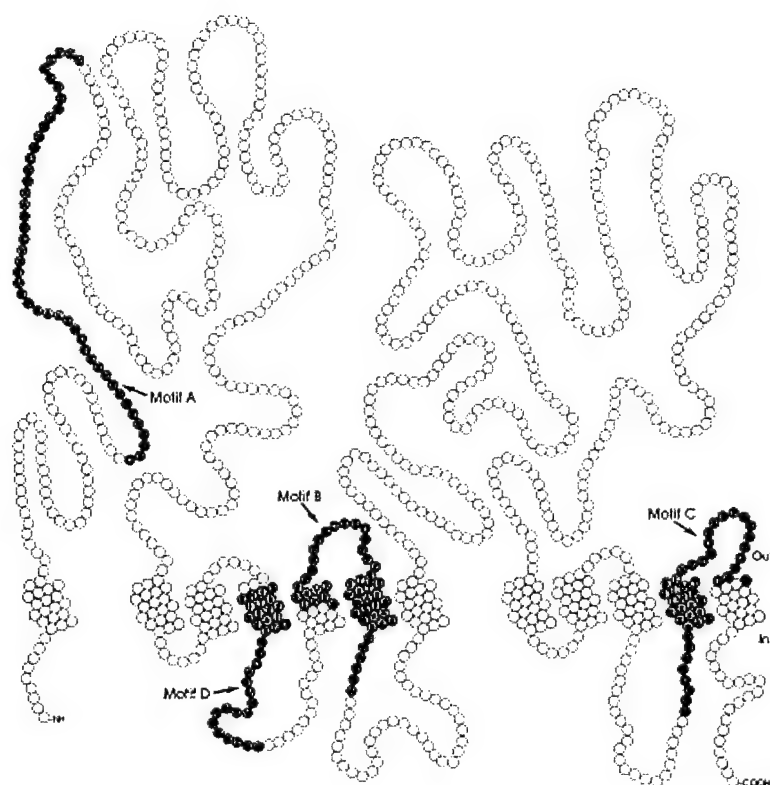


Figure 1.3. Structural Model of the Cytoplasmic Membrane Component of an RND Multidrug Transporter. These components of RND efflux pumps contain 12 transmembrane segments that transverse the cytoplasmic membrane. There are two large periplasmic loops between segments 1 and 2 and between segments 7 and 8. These loops supposedly form an internal channel through which a compound can be delivered to the membrane fusion lipoprotein. The highlighted motifs represent homology among the RND transporters (52, 57, 76).

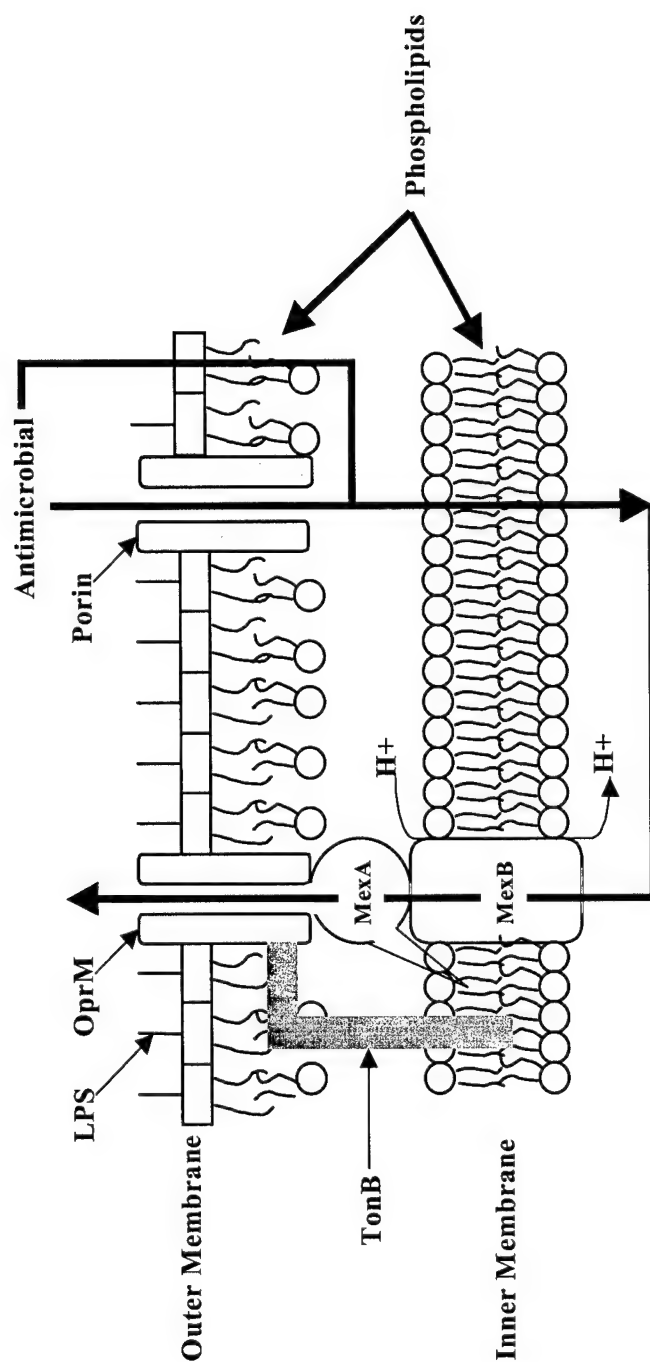


Figure 1.4. The MexAB-OprM RND Efflux Pump of *P. aeruginosa*. Antimicrobials slowly penetrate the *P. aeruginosa* outer membrane permeability barrier by simple diffusion either through a porin or the outer membrane. Antimicrobials that reach the cytoplasm are pulled into the RND transporter MexB, which is powered by the PMF. Compounds are expelled in an antiport fashion where protons enter the cell and the compound exits. It is then delivered to MexA, a MFP. MFP structure is thought to be a function of its substrate and is therefore just as divergent as RND proteins. TonB is a PMF transducer. It opens and closes the gated channel OprM by undergoing a conformational change which elicits a conformational change in OprM (36, 52, 55, 57, 68, 78). The peptidoglycan layer in the periplasm is not shown.

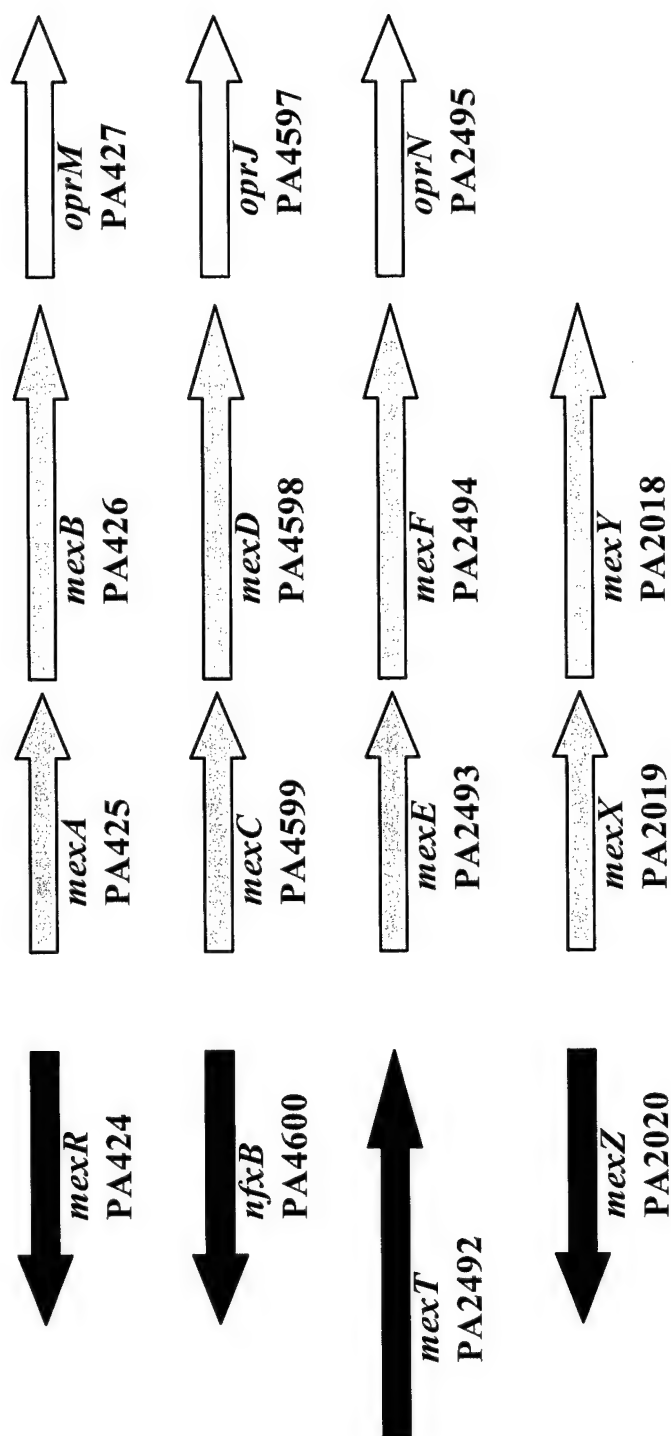


Figure 1.5. Characterized Multidrug Efflux Pumps of *P. aeruginosa*. The regulatory genes *mexR*, *nfxB*, and *mexZ* are all divergently transcribed from their respective operons. Their products negatively regulate/repress the expression of their corresponding efflux operons. The *mexT* gene is transcribed convergently and its product regulates the *mexEF-oprN* efflux operon by positive regulation. Interestingly, the *MexXY* pump does not have its own outer membrane protein channel, but instead probably uses *OprM* (1, 11, 33, 54, 55, 75).

P. aeruginosa is significantly more drug resistant than other bacteria because of the synergy achieved by its outer membrane permeability barrier, and its myriad of efflux pumps (38). MexAB-OprM was the first RND efflux pump discovered in *P. aeruginosa*, and is the most widely studied (55). It is highly conserved and constitutively expressed in wild type strains of *P. aeruginosa*, and homologues have been found in *P. aureofaciens*, *P. chlororaphis*, *P. syringae*, and *P. putida* (7). It was discovered by Dr. Keith Poole et al. of Queens University in 1993 by complementation of a siderophore-deficient mutant (55). MexAB-OprM was suspected in secreting a high affinity iron chelator called pyoverdine, which is one of two siderophores in *P. aeruginosa*. Siderophores are commonly secreted from bacteria to complex iron so that it can be taken up by the cell via a receptor. In the same study, it was shown that MexAB-OprM expressing strains were resistant to ciprofloxacin, nalidixic acid, tetracycline, chloramphenicol, and streptomycin. (53, 55).

Ironically, MexAB-OprM and MexEF-OprN may reduce *P. aeruginosa* virulence by interfering with its cell-to-cell communication system called quorum sensing (17, 34) (figures 1.6 and 1.7). Before reviewing *P. aeruginosa* infections and virulence, a brief review of its microbiology will be presented.

1.3 MICROBIOLOGY OF *PSEUDOMONAS AERUGINOSA*

P. aeruginosa is a rod shaped, gram negative, motile, aerobic, opportunistic pathogen thriving ubiquitously in the environment (figure 1.1). Its tenacious nature to inhabit nearly everything is attributed to its large array of enzymes that permit it to adapt successfully in diverse environments.

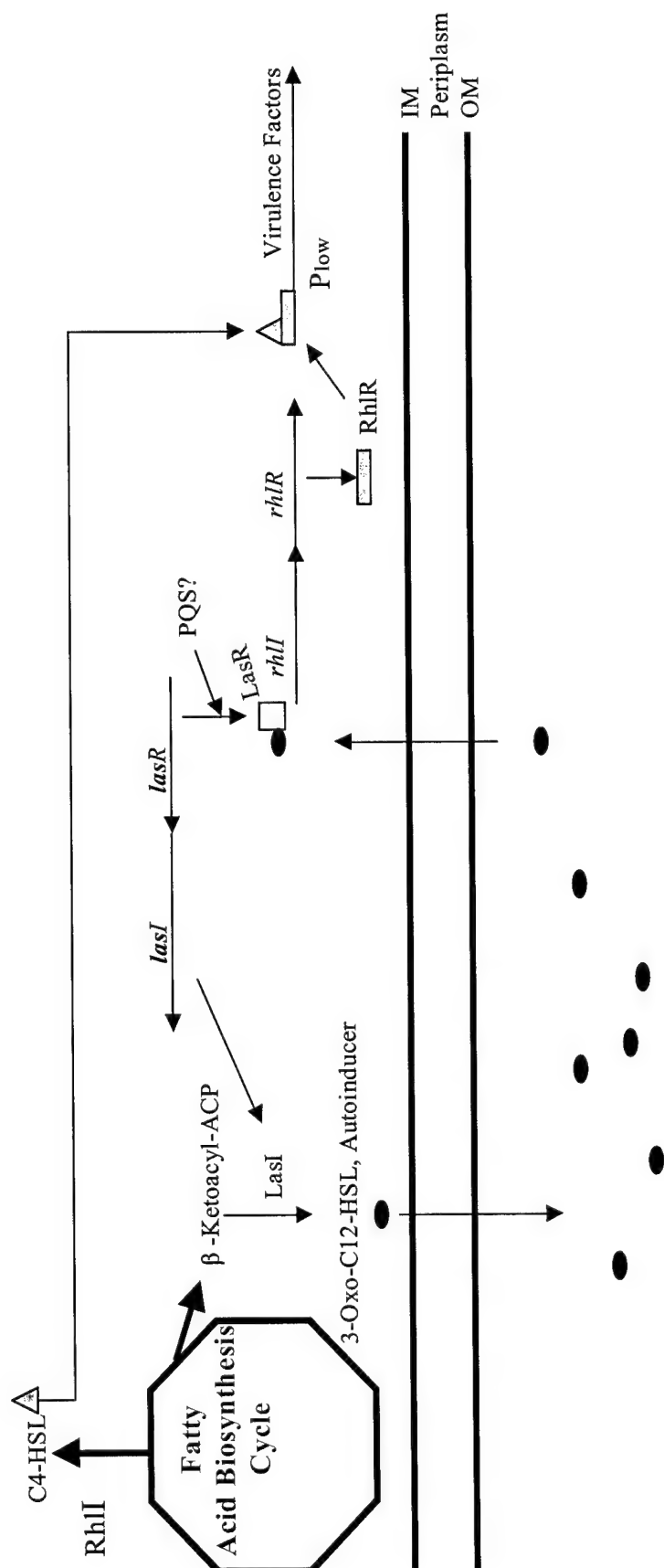


Figure 1.6. HSL Mediated Cell-to-Cell Communication in *P. aeruginosa*. From left to right: 3-oxo-C12-Acylated HSL (Acyl-HSL) is generated from the fatty acid biosynthesis cycle from β-ketoacyl-ACP and LasI which is an autoinducer synthase. The autoinducer (AI) is secreted out of the cell where it mingles in the general area of the bacterium. Once enough bacterial cells have reached a specific density or "quorum," enough AI exist to reenter the bacterium to bind with the Las System Regulator, indicated as a box. This complex then facilitates the expression of *rhlI*, encoding another AI synthase which is responsible for catalyzing the synthesis of C4-HSL at the top of the FAB cycle and is indicated as a triangle. Again, once enough of the C4-HSL is transported back into the cell, it complexes with RhlR (rectangle). This complex facilitates the expression of *P. aeruginosa* virulence factors such as pyocyanin, elastase, and rhamnolipids (27, 34)

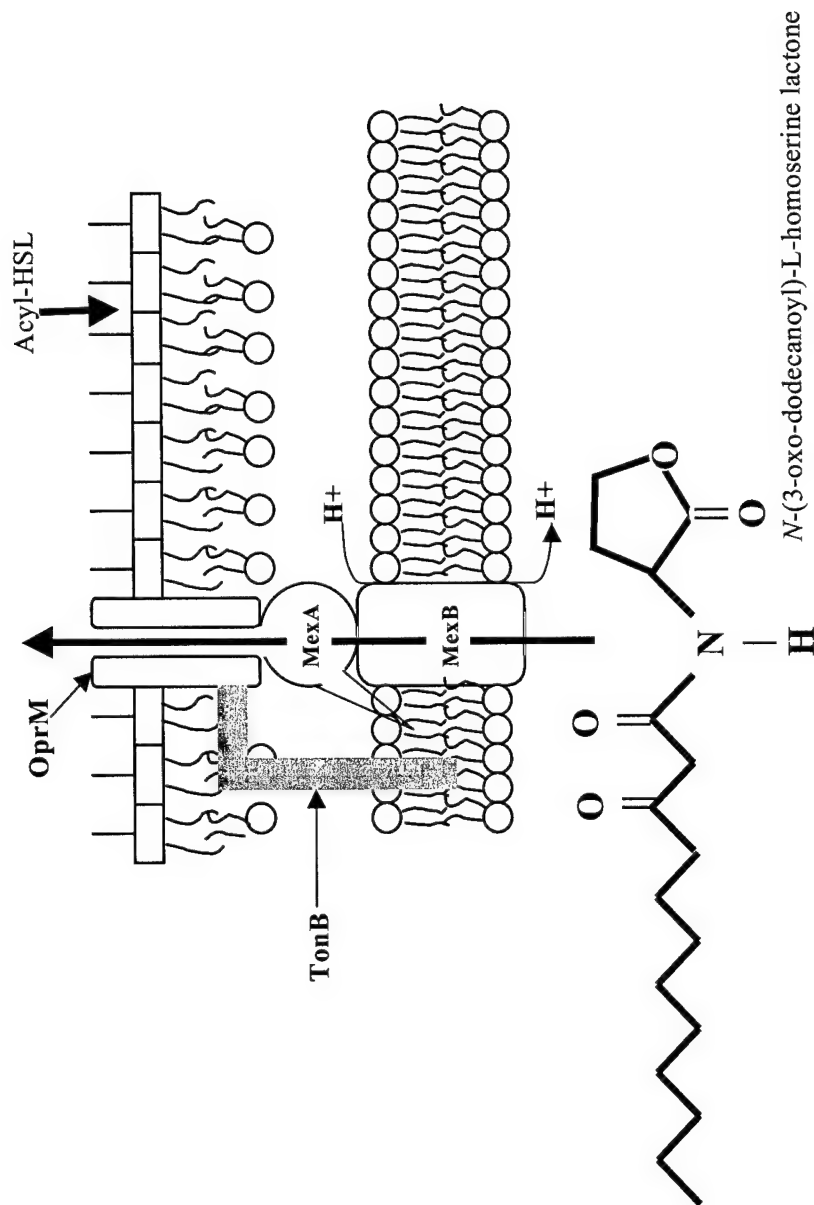


Figure 1.7. MexAB-OprM Mediated Autoinducer Efflux. Acyl HSL enter the cytoplasm by simple diffusion through the cell envelope, presumably facilitated by their acyl tails. It has been suggested that the MexAB-OprM and MexEF-OprN efflux pumps may efflux HSLs before they have an opportunity to complex with their respective regulators and facilitate transcription of various virulence factors. *Pseudomonas* quinolone signal (PQS), which maybe an intermediary between the *las* and *rhl* quorum sensing systems may also be effluxed (17, 34).

It has been found in surgical scrub water, swimming pools, distilled water, soil, and can even colonize stainless steel (10). It can normally be found in soil and biodegrading organic waste, which may contribute to its ability to resist antibiotic treatment. Interestingly, researchers are attempting to exploit *P. aeruginosa's* bioremediation capabilities in toxic waste clean-up efforts (10). Rhamnolipids, for instance, are biosurfactants secreted by *P. aeruginosa* which cause enhanced cell surface hydrophobicity (2, 3). Because rhamnolipids lower cell surface tension, they can disperse as emulsions in water or other liquids, making them an excellent medium for oil recovery (35).

Although *P. aeruginosa* was first described in the late 1800's, it has only recently become the nosocomial pathogen that it is today. Its widespread emergence in hospitals correlates to its innate ability to resist antibiotic treatment, and increasing numbers of susceptible hosts in industrialized nations (9).

Its opportunistic nature lends itself to victims with a depressed immune system such as patients with AIDS, cancer patients undergoing chemotherapy, and patients of advanced age. *P. aeruginosa* also causes infections in patients who have sustained thermal injuries in which the first line of the innate immune system, the skin, is critically damaged (29). *P. aeruginosa's* role in human medicine is most important in patients with cystic fibrosis (CF) (20).

Table 1.2
***P. aeruginosa* Virulence Factors^a**

Virulence Factor	Physiological Role
Alginate	Helps to form biofilms, interferes with opsonization, which is critical for phagocytosis, no activation of the alternate complement pathway and thus not only inhibits bacterial lysis but also PMN ^b chemotaxis.
Elastase	Coded by <i>lasB</i> , secreted proteolytic enzyme, degrades antibiotics, inactivates human gamma interferon, causes direct tissue damage, and inactivates C3a and C5a of the complement cascade.
Exotoxin A	Protein toxin of the adenosine diphosphate-ribosylating toxin (ADPRT) family, LD ₅₀ for mice is 2.5 µg/kg making it the most toxic compound generated by <i>P. aeruginosa</i> , targets eukaryotic elongation factor 2 by binding NAD and linking its ADP-ribose moiety. This inhibits protein synthesis.
Exoenzyme S	Also an ADPRT, however its precise role is currently unknown, may play a role in adhesion and erythrocyte agglutination
Lipopolysaccharide	Permeability barrier to hydrophilic compounds
Rhamnolipid	Inhibits mucociliary transport, cytolytic effect on monocyte-derived macrophages by unknown mechanism(s) reduces macrophage's ability to bind bacteria thereby inhibiting phagocytosis.

^aCompiled from (9, 10, 20, 42)

^bPolymorphonuclear cells

1.4 VIRULENCE, INFECTIONS, AND CLINICAL SIGNIFICANCE

P. aeruginosa is armed with virulence factors ranging from specialized surface structures to exotoxins (table 1.2). Of all of these virulence factors, the most important in CF cases is alginate (10). The secretion of alginate allows *P. aeruginosa* to assume its mucoidal phenotype, which allows it to colonize in aqueous environments such as the lungs (20). To understand the significance of such infections it is first necessary to understand the pathogenesis of CF, and the host immune response to *P. aeruginosa* infections.

CF is caused by a mutation in the cystic fibrosis transmembrane regulator (CFTR) responsible for the regulation of chloride ion transport in cells (20). Ironically, CFTR belongs to the same family of transporter proteins as the efflux pump P-glycoprotein that commonly effluxes chemotherapeutic drugs from cancer cells (table 1.1). Patients homozygous for this mutation suffer from CF, and people heterozygous are carriers. The incidence of homozygous CF in Caucasian populations is 1 in 2500 and 1 in 25 in the heterozygous condition, making it the most common inherited lethal disorder in Caucasians (20). The improper balance of chloride ion in CF patients cause the cell lining in internal organs to amass a sticky dehydrated mucus in the airways of the lungs. This in turn disrupts the normal mucociliary export of bacteria and otherwise foreign particles out of the airway. This is the opportunity *P. aeruginosa* seizes to set up chronic infections, which ultimately lead to damaging pulmonary exacerbations, permanent lung damage, respiratory arrest, and death (10, 20).

To answer the question of why such a mutation is so prevalent, heterozygous mice were engineered and studied. It has been shown that these mice are significantly resistant

to *Vibrio cholerae* toxin. Cholera toxin deregulates electrolyte transport in the intestines, often times causing fatal diarrhea. If heterozygous humans were also more resistant to this disease, it has been argued that such a mutation would be advantageous to the population as a whole (19).

Normally, when *P. aeruginosa* finds its way into a healthy host, opsonizing antibodies immediately bind to its outer surface structure. Macrophages are then enabled to readily identify the invaders via Fc receptors and rapidly phagocytose them, exposing them to lysozyme, nitric oxide and other toxic oxygen radicals. Furthermore, most invading bacteria are normally trapped in a thick mucous layer that surrounds the lumen of air ducts. An upward ciliary movement takes the contaminated mucous out of the respiratory system into the larynx where it may be expelled or swallowed. Hence, most bacteria are never given an opportunity to set up an infection (30).

In CF patients this mucous layer becomes viscous and dehydrated, and is therefore rendered nonfunctional (20). Invading *P. aeruginosa* rapidly synthesize alginate, a mucopolysaccharide capsule, that aids in the formation of biofilms, and ultimately enables them to colonize the lungs. Bacterial colonization causes a massive influx of neutrophils and other PMNs resulting in intense inflammation and irreversible damage to the lungs. Typical complications arise immediately such as bronchiolitis, pneumothorax, and fibrosis. Over time these conditions manifest into respiratory failure and death. *P. aeruginosa* is the “most common pathogen infecting CF patients...and is the most important and controversial new opportunistic pathogen to challenge microbiologists” (20).

1.5 TRICLOSAN

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether), also known as Irgasan CH3565, is a synthetic, nonionic, bisphenol biocide (6) (figure 1.8). It is an off white, odorless, and tasteless powder (6). Due to its low toxicity, broad-spectrum antimicrobial capabilities and its thermal stability, triclosan has been the most widely used bisphenol for the past thirty years (6, 43).

It is usually found in hand soaps, surgical scrubs, deodorant soaps, and toothpastes. It is also routinely incorporated into many different plastics ranging from children's toys to mop handles in order to protect the plastic from biodegradation (31). Until recently, triclosan was used primarily in hospitals in an effort to prevent nosocomial infections (12, 43).

Triclosan is normally taken up by the cell via diffusion (59). Its biocidal activity was originally thought to operate in a nonspecific manner, which is characteristic of antiseptics. However, it has been recently shown to specifically target FabI, an enoyl-acyl carrier protein reductase, in *P. aeruginosa* and *fabI* homologs in other bacteria (23, 26, 45, 67).

It is widely accepted that *P. aeruginosa* is intrinsically resistant to triclosan. In fact, biotech companies such as Difco sell *Pseudomonas* isolation agar (PIA), which contains 25 µg/ml of triclosan (15). *P. aeruginosa*'s resistance to triclosan was initially thought to be caused by the low permeability of its cell wall. Its resistance to triclosan was later discovered to be caused by MexAB-OprM, which is constitutively expressed in *P. aeruginosa* strains (11, 55).

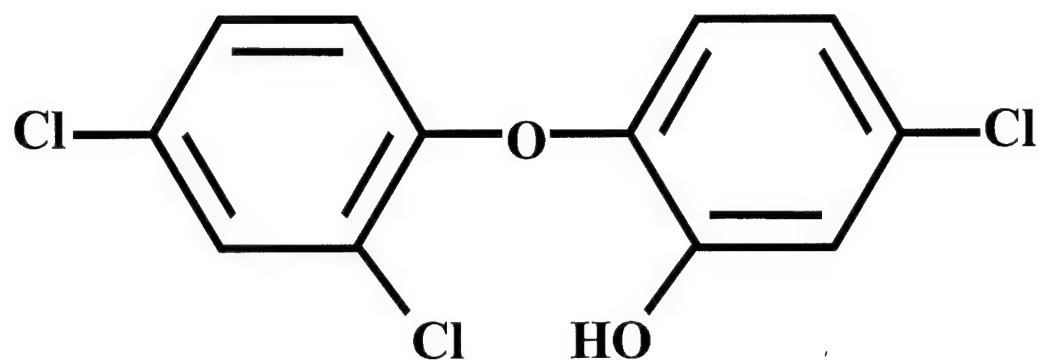


Figure 1.8. Triclosan. Triclosan is a nonionic, tasteless, off white, synthetic bisphenol biocide common in toothpastes, hand soaps, and plastics. Another bisphenol biocide, hexachlorophene, has been limited in consumer products due to its questionable toxicity (59).

For years concerns have mounted in the scientific community over the possibility of cross resistant bacterial strains emerging under antiseptic pressure. In 1992 Masuda et al. isolated PAO1 mutants from ofloxacin and cefsulodin containing agar plates (41). These mutants hyperexpressed OprM and as a result became resistant to meropenem, cepheems, and quinolones. Triclosan has also been shown to rapidly select for mutants in a MexAB-OprM deletion mutant by selecting for mutations in the regulator region of MexCD-OprJ (12). These MDR mutants were significantly resistant to tetracycline, trimethoprim, ciprofloxacin, and erythromycin (12).

1.6 MexJK Efflux Pump

The *mexJK* efflux operon and *mexL* were discovered through a series of experiments accomplished by Chuanchuen et al. (12). The *mexAB-oprM* operon was knocked out in PAO1, which resulted in the antibiotic sensitive strain, PAO200. PAO200 was plated on PIA, and resistant colonies emerged that expressed the MexCD-OprJ efflux pump (12). These were MDR strains of *P. aeruginosa*, and were designated PAO200-1, 2, 3, and 4. Hence, triclosan was shown to select for mutations in *P. aeruginosa* efflux pump regulator genes that caused cross resistance to medically important antibiotics. This is alarming due to the ubiquitous use of triclosan, and inherent antibiotic resistant mechanisms of *P. aeruginosa*. Chuanchuen et al. (12) proceeded to delete *mexCD-oprJ* from PAO200 that resulted in the double knockout mutant, $\Delta(mexAB-oprM) \Delta(mexCD-oprJ)$, named PAO238. PAO238 was also plated on PIA, and like PAO200 resistant colonies emerged. These strains were named PAO238-1, 2, 3, and 4 (12).

The MexEF-OprN efflux pump was ruled out as the causative resistance mechanism in PAO238-1 when Western blot analysis failed to demonstrate the expression of OprN. It was later discovered that PAO1, the parent strain of PAO238, is itself a double *mexEF-oprN* mutant (12, 32, 40). MexT is a positive regulator of the *mexEF-oprN* operon, and MexS is a negative regulator of *mexT*. When MexT was introduced to PAO238, OprN was detected. This suggested that *mexS* and *mexT* are both mutated in PAO1 (12).

Much of the physiology of the MexXY efflux pump is hitherto unknown. It was concluded, however that MexXY was not expressed in PAO238-1 when Western blot analysis failed to reveal MexX (12). However, The genes that code for MexXY are still present in the chromosome. Furthermore, the MexXY efflux pump requires OprM to efflux antibiotics (12).

Since triclosan resistance was not caused by any of the hitherto characterized efflux pumps of *P. aeruginosa*, it was reasoned that a new efflux pump may be involved. This efflux pump, the fifth RND pump of *P. aeruginosa*, was characterized and named MexJK. Further analysis of PAO238-1 revealed a single base pair mutation in a gene, *mexL*, that is divergently transcribed from the *mexJK* operon and encodes a protein belonging to the TetR family of repressor proteins. This mutation caused a change from an alanine, a nonpolar amino acid, to an aspartic acid, an acidic amino acid, in the first turn of the putative helix-turn-helix region of MexL, which supposedly prevented the proper binding of MexL in the promoter region of the *mexJK* operon. This resulted in the constitutive expression of the *mexJK* operon, which ultimately led to triclosan resistance in strain PAO238-1 (12).

1.7 RESEARCH AIMS

The purpose of this study was to determine the regulatory mechanism(s) of a newly characterized RND efflux pump, MexJK, in *P. aeruginosa* (figure 1.9). A gene, designated *mexL*, encoding a protein that belongs to the TetR family of repressor proteins is divergently transcribed, and is located 94 bp upstream of the *mexJK* operon.

The hypothesis is that MexL is a transcriptional repressor of the MexJK efflux pump, and thus inhibits the expression of *mexJK* by binding via its putative helix-turn-helix motif in the promoter containing region of the *mexJK* operon.

The specific aims of this study were:

- 1) To engineer a Δ *mexL* mutation and characterize it phenotypically and genetically.
- 2) To study MexL transcriptional regulation in *P. aeruginosa* and *E. coli* by engineering *lacZ* operon fusion strains.

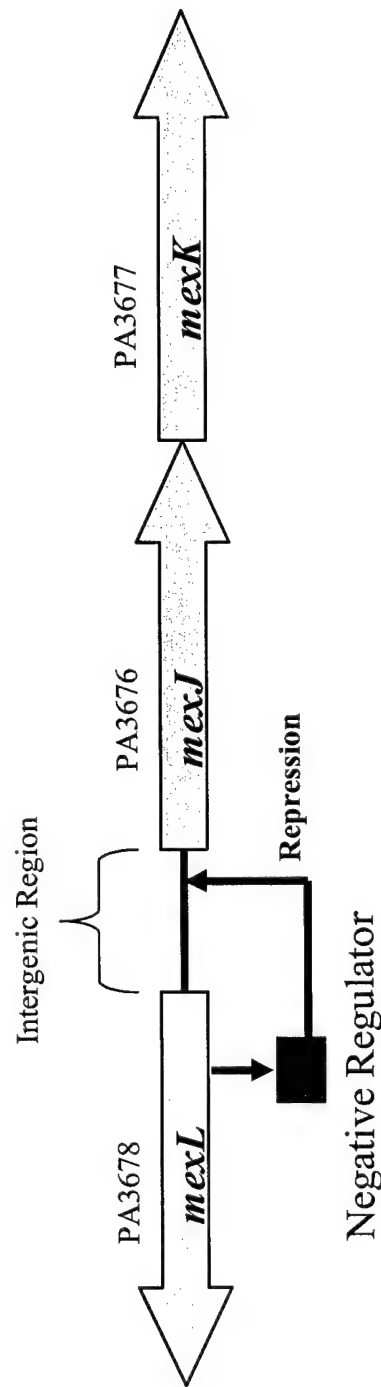


Figure 1.9. Organization of *mexL* and the *mexJK* Efflux Pump Operon. This efflux system is interesting in two obvious respects: [1] It lacks its own outer membrane protein, and [2] The 94 bp intergenic region where *mexL* and *mexJK* regulation occurs is relatively small. It has been shown by Chuanchuen et al. that this pump requires OprM to efflux clinically significant antibiotics. However, OprM is not required for MexJK to efflux triclosan. It is currently unknown if triclosan efflux is using a hitherto uncharacterized outer membrane protein, or if triclosan simply is effluxed to the periplasm. The PA numbers show the annotation given by the genome sequencing group.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS, PLASMIDS, AND OLIGONUCLEOTIDES

Bacterial strains, plasmids and oligonucleotides used in this project are listed in tables 2.1, 2.2, and 2.3 respectively. *E. coli* DH5 α F' was used for standard transformations unless otherwise indicated. *E. coli* SM10, a plasmid mobilizing strain, was used for all conjugations. For long-term storage, φ strains were grown overnight, as indicated below, mixed with sterile glycerol to a final concentration of 15%, and stored at -70°C .

2.2 MEDIA AND GROWTH CONDITIONS

All growth media were autoclaved at 121°C (20 psi) for at least 15 min prior to use. All cultures contained the appropriate filter or alcohol sterilized antibiotics at the final concentrations listed in table 2.4. In the case of agar plates, antibiotics were added when the agar reached handwarm temperatures. Unless otherwise specified, all broth cultures were incubated O.N. in 4 ml of Luria Broth (LB) in a 37°C shaker, and plated cultures were incubated on LB mixed with 1.5% agar in a 37°C incubator.

Table 2.1
Bacterial Strains

Strain	Genotype	Reference/Source
<i>E. coli</i>		
DH5 α F'	[F ⁺ Φ 80 Δ <i>lacZ</i> Δ M15] Δ (<i>lacZYA-argF</i>)U169 <i>recA1</i> <i>endA1</i> <i>hsdR17</i> [r _K ⁻ m _K ⁺] <i>supE44</i> <i>thi-1</i> <i>gryA</i> <i>relA1</i>	(39)
SM10	<i>thi thr leu tonA lacY</i> <i>SupE</i> <i>recA::RP4-2Tc::Mu</i> Km ^r	(66)
DL291	<i>recA⁻ ΔlacZ</i>	Schweizer Lab Collection
MC4100	F ⁻ (<i>argF - lac</i>) U169 <i>araD139</i> <i>rpsL 150</i> <i>relA1</i> <i>thiA</i> <i>ptsF25</i> <i>deoC1</i> <i>flbB5301</i> <i>rbsR</i>	Schweizer Lab Collection
JM110	<i>rpsL thr leu thi-1 galK galT ara tonA tsx dam dcm sup</i> <i>E44</i> <i>relA1</i> Δ (<i>lac-proAB</i>)/F' <i>traD36</i> <i>proAB⁺</i> <i>lacI^q</i> <i>lacZ</i> Δ M15	Stratagene
λ Mex	DL291 lysogenized with λ RZ5 <i>PmexJ-lacZ</i> Km ^r	This Study
<i>P. aeruginosa</i>		
PAO1	prototrophic strain	(28)
PAO238	PAO1 Δ (<i>mexAB-oprM</i>) Δ (<i>mexCD-oprJ</i>)	(12)
PAO238-1	Spontaneous Tri ^r mutant derived from PAO238	(12)
PAO314	PAO238-1 Δ (<i>mexJKL::FRT</i>)	(12)
PAO318	PAO238 Δ (<i>mexL::FRT</i>)	This Study
PAO319	PAO238 Δ (<i>mexL::FRT-Gm^r-FRT</i>)	This Study
PAO320	PAO314 with chromosomal <i>PmexJ-lacZ</i>	This Study
PAO321	PAO314 with chromosomal <i>mexL</i> mutant gene and <i>PmexJ-lacZ</i>	This Study
PAO323	PAO314 with chromosomal promoter-less <i>lacZ</i> gene	This Study

Table 2.2
Plasmids

Plasmid	Properties	Reference/Source
mini-CTX3- <i>lacZ</i>	Cb ^r ; Gm ^r ; <i>int</i> ; suicide integration vector	(62)
pBluescript II SK(-)	Ap ^r ; cloning and sequencing vector	Stratagene
pBSP II SK(-)	Cb ^r ; broad-host-range derivative of pBluescript II SK(+/-)	(61)
pEX18Ap	Cb ^r ; <i>oriT</i> , <i>sacB</i> ; suicide cloning vector	(24)
pFLP2	Cb ^r ; source for Flp recombinase; <i>sacB</i> , <i>oriT</i>	(24)
pJ22	Cb ^r ; pADD948 with ~32 kb chromosomal insert from PAO238-1	(12)
pPS856	Cb ^r ; Gm ^r ; source of <i>FRT</i> -Gm ^r cassette	(24)
pPS1150	Cb ^r ; pBSP II SK(+) with 6,945 bp <i>NotI</i> fragment from pJ22	(12)
pPS1153	Cb ^r ; pUCP20T with 816-bp <i>mexL</i> fragment	(12)
pPS1175	Cb ^r ; pBluescript SK (-) with 1,342-bp <i>EcoRI</i> - <i>ClaI</i> fragment from pPS1150	This Study
pPS1176	Cb ^r ; deletion of 725-bp <i>XhoI</i> fragment from pPS1175	This Study

Table 2.2 Continued Plasmids		
Plasmid	Properties	Reference/Source
pPS1177	Gm ^r ; mini-CTX3- <i>lacZ</i> with 643-bp <i>XhoI</i> - <i>PstI</i> fragment from pPS1176	This Study
pPS1179	Gm ^r ; mini-CTX3- <i>lacZ</i> with 1,348-bp <i>SalI</i> - <i>PstI</i> fragment from pPS1175	This Study
pPS1181	Cb ^r ; pTZ110 with 655-bp <i>Bam</i> HI- <i>XhoI</i> fragment from pPS1176	This Study
pPS1182	Cb ^r ; Gm ^r ; pEX18Ap with 1,348 bp <i>PstI</i> - <i>SalI</i> fragment from pPS1192	This Study
pPS1192	Cb ^r ; pPS1208 with Gm ^r - <i>FRT</i> cassette from pPS856	This Study
pPS1193	Km ^r ; pUC19Km mini-CTX- <i>PmexJ</i>	This Study
pPS1206	Km ^r ; pUC19Km	This Study
pPS1208	Cb ^r ; deletion of <i>XhoI</i> site from pPS1175	This Study
pTZ110	Cb ^r ; broad-host-range <i>lacZ</i> fusion vector	(63)
pUCP20T	Ap ^r ; pUCP18-derived broad-host range cloning and sequencing vector with cloned RK2-derived <i>oriT</i>	(64)
pUC19	Ap ^r ; cloning and sequencing vector	(77)
pUC4K	Cb ^r ; Km ^r ; source of Km ^r cassette	(71)

Table 2.3 Oligonucleotides	
Name	Sequence
<i>mexL</i> -up ^a	5' – ACTGGG TCG ACCACTGGGACATC - 3'
<i>mexL</i> -down	5' – CGTTC GAATT CTTATACTGGGCGG - 3'

^a These oligonucleotides contain base changes from the original sequence that introduce *Sal*I (*mexL*-up) and *Eco*RI (*mexL*-down) into the resulting PCR fragment. Restriction sites are indicated in bold-face letters.

Table 2.4
Antibiotics – Preparation and Use

Antibiotic	Abbreviation	Stock Conc. (SC) mg/ml	Solvent	SC storage °C	Working Concentrations <i>E. coli</i>	Working Concentrations <i>P. aeruginosa</i>
Acriflavin	Acr	100	dH ₂ O	RT	N/A ^a	* ^b
Ampicillin	Ap	100	dH ₂ O	-20	100	N/A
Carbenicillin	Cb	100	dH ₂ O	-20	N/A	100-200
Chloramphenicol	Cm	25	95% Ethanol	-20	N/A	*
Dichlorophene	Dp	100	95% Ethanol	-20	N/A	*
Erythromycin	Em	50	95% Ethanol	-20	N/A	*
Fusidic Acid	Fus	100	95% Ethanol	4	N/A	*
Gentamycin	Gm	40	dH ₂ O	-20	15-25	5-15
Hexachlorophene	Hp	10	95% Ethanol	-20	N/A	*
Kanamycin	Km	35	dH ₂ O	4	15	N/A
Tetracycline	Tc	10	70% Ethanol	-20	N/A	*
Trimethoprim	Tp	100	Dimethylacetamide	-20	N/A	*

^a N/A - Not applicable, these antibiotics were not used in the indicated organism

^b * - Antibiotics used to determine working concentrations of PAO318 and PAO319 via MIC (see table 3.1)

LB was purchased from Gibco-BRL (Gaithersburg, MD). One liter of LB contained 10 g tryptone, 5 g yeast extract, and 5 g NaCl.

Vogel and Bonner Medium (VBMM) was used to counter-select against *E. coli* after biparental matings between *E. coli* and *P. aeruginosa* strains, because *E. coli* is unable to utilize citrate as its only carbon source. However, *E. coli* can reside latently on a VBMM plate. Hence, isolation streaking of *P. aeruginosa* colonies on VBMM plates 24 h after conjugation was routinely accomplished to avoid *E. coli* contamination during subsequent inoculations. One liter of VBMM contained 3 g citric acid Na₃ salt, 2 g citric acid, 10 g K₂HPO₄, and 3.5 g Na₂NH₄PO₄ × 4 H₂O (74).

Mueller-Hinton (MH) broth and agar plates were used for minimum inhibitory concentration (MIC) determinations as specified by the National Committee for Clinical Laboratory Standards (48). MH broth was used within one week of preparation, and was cation adjusted to contain between 20-25 mg of Ca²⁺ and 10-11.5 mg of Mg²⁺ per liter. MH broth was stored at 4°C, and was checked for a pH of 7.2-7.4 at 25°C. It was purchased from Difco (Detroit, MI). One liter of MH broth contained 2 g of beef extract powder, 17.5 g acid digest of casein, and 1.5 g soluble starch. E-Test plates were prepared by pouring exactly 25 ml of warm MH agar into a 100 × 15 mm petri dish and allowed to gel on a flat surface. They were used within 24 h of preparation.

Minimal Medium (M9) was used for O.N. cultures grown for β-Galactosidase assays. One liter of 5 × M9 contained 30 g Na₂HPO₄, 15 g KH₂PO₄, 2.5 g NaCl, and 5 g NH₄Cl. 50 ml aliquots were taken as needed and diluted with dH₂O to make 250 ml of 1 × M9. To these 0.0125 ml of sterile 1 M CaCl₂ and 0.0625 ml of sterile 1 M MgSO₄ were added. It was supplemented with filter sterilized 20% casamino acids as a carbon

source to a final concentration of 0.2% just prior to use.

MacConkey agar plates were used to select for *lacZ* containing *E. coli* lysogens. One liter of MacConkey agar contained 17 g of peptone from casein, 3 g peptone from meat, 5 g NaCl, 10 g lactose, 1.5 g bile salt mixture, 0.03 g neutral red, 0.0001 g crystal violet, 13.5 g agar-agar, and was adjusted to pH 7.1 \pm 0.1 at 25⁰C. It was purchased from BDH (Darmstadt, Germany).

X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was purchased from Gold BioTechnology (St. Louis, MO). It was dissolved in *N,N*-dimethyl formamide to a final concentration of 20 mg/ml and was stored at -20⁰C. Before plating bacteria, 50 μ l of this stock was applied to the center of the plate into a droplet of 100 μ l of dH₂O to a final concentration of 40 μ g/ml (one agar plate contained ~25 ml of media). This solution was spread evenly and aseptically across the surface of the agar plate. In some instances, X-Gal stock was added directly to warm top agar to give a final concentration of 40 μ g/ml. X-Gal was used to select *lacZ*⁺ and *lacZ* strains of *E. coli* and *P. aeruginosa* throughout this project.

Tryptone Broth (TB) top agar was used for isolating and calculating the spontaneous excision frequency of bacteria phage λ RZ5. It contains 0.7 g of agar in 100 ml of TB broth. 500 ml of TB broth contained 10 g Bacto Tryptone, 5 g NaCl, and 5 ml 1 M MgSO₄. Phage λ dilution buffer was used for serial dilution of λ lysates. 100 ml contained 1 ml 1 M Tris-HCl (pH 7.5) and 0.5 ml 1 M MgSO₄.

2.3 DNA TECHNIQUES

2.3.1 Isolation of Plasmid DNA

Plasmid DNA was routinely isolated from *P. aeruginosa* and *E. coli* using QIAprep Spin Miniprep Kit purchased from Qiagen (Valencia, CA). This protocol is modified from the alkaline lysis method of Birnboim and Doly (8). Briefly, bacterial cell membranes are lysed by SDS, which solubilizes the phospholipid and protein components of the cell membrane. Under alkaline conditions, established in the same step in the protocol, lysis of genomic DNA and proteins occur. The addition of a subsequent buffer, N3, neutralizes, and adjusts the sample to high-salt binding conditions, which causes denatured proteins, chromosomal DNA, cellular debris, and SDS to precipitate. Plasmid DNA renatures correctly and is subsequently bound to a silica-gel membrane. Salts left on the silica gel membrane with plasmid DNA are washed away by buffer PE. Finally, plasmid DNA is eluted with dH₂O or Buffer EB (8).

These preparations were usually digested (section 2.3.3) and then electrophoresed on a 0.8%-1.5% agarose gel prepared in 1 x Tris-acetate-EDTA (TAE) buffer and ran at 80-90 V for 45-60 min respectively (58). Ethidium bromide, 0.5 µg/ml, was either included into the agarose matrix or added on the anode side of the running buffer prior to applying voltage to the system. Eight to ten microliters of Hi-Lo DNA marker (Minnesota Molecular) and 6 x DNA loading buffer was used during electrophoreses. Plasmid preparations that were to be used for electroporation experiments were eluted with 30-40 µl of warm dH₂O in place of elution buffer provided for in the preparation kit.

2.3.2 Isolation of Chromosomal DNA

Chromosomal DNA was isolated for PCR reactions and Southern Blotting Assays (sections 2.3.6 and 2.9). Isolation was accomplished using the IsoQuick Nucleic Acid Extraction Kit from Orca Research, Inc. (Bothell, WA). Chromosomal DNA was stored at 4⁰C. A portion of these samples were diluted 1:50 in dH₂O and assayed in a spectrophotometer at 260 nm. DNA concentrations were calculated using the following equation (5):

$$\text{DNA } \mu\text{g/ml} = (A_{260\text{nm}})(\text{Dilution Factor, 50}) [(50\mu\text{g/ml})/1 A_{260\text{nm}}]$$

2.3.3 Restriction Digests

Restriction digests were accomplished using enzymes purchased from Gibco-BRL. Restriction digests were accomplished by combining: 14.5 μ l dH₂O, 2 μ l appropriate 10 x REact buffer, 3 μ l DNA depending on its concentration, and 0.5 μ l restriction endonuclease. These reactions were briefly vortexed in a 1.5 ml microfuge tube, spun down, and incubated at 37⁰C for 1 hour. The volume of dH₂O added was relative to the volume of DNA added. When two endonucleases were used for digestions that required different REact (reaction) buffers, the most compatible buffer and its corresponding enzyme were used first. After 1 h of incubating at 37⁰C the next enzyme and buffer were introduced to the reaction mixture, and incubated for an additional 30-60 min.

Blunt-ending was accomplished by adding 1 μ l of 2 mM dNTP and 0.5 μ l of T4 DNA polymerase directly to the digestion reaction and incubated for 30 min at room

temperature. An agarose gel was run to free the mixture of T4 polymerase and dNTPs prior to using the DNA for ligation reactions (section 2.3.5).

2.3.4 Extraction of DNA From Agarose Gels

DNA fragments were routinely isolated from agarose gels using a QIAquick Gel Extraction Kit provided by Qiagen. Briefly, the desired DNA fragment was cut out of the gel resulting in a gel segment, and was placed in a clear 1.5 ml microfuge tube. Reagents were added to break down the gel matrix and free the DNA. The DNA was then bound to a silica-gel membrane filter under high salt concentrations at a $\text{pH} \leq 7.5$. Impurities such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils, and detergents were washed through the membrane with an ethanol-containing buffer. Finally, DNA was eluted into a sterile microfuge tube using 30-50 μl of warm 10 mM Tris-HCl at pH 8.5. DNA was stored at -20°C .

2.3.5 Ligations

Ligations were accomplished in a 1:5 and 1:10 (vector:insert) molar ratio. DNA concentrations were estimated by comparing DNA band intensities on the agarose gel before band extraction to a known standard. Ligations were accomplished by combining 5 μl vector, 10 μl insert, 4 μl 5X T4 ligase buffer, and 1 μl of T4 DNA ligase. Sticky end ligation reactions were incubated for 3 h at room temperature prior to transformation into *E. coli* DH5 α F' cells. Blunt end ligation reactions were incubated for 24 h at 14°C prior to transformation. A vector control, lacking the insert, was always ran in conjunction with the 1:5 and 1:10 ligation reactions. Ligations were verified using

restriction digest and in some cases PCR. T4 ligase and buffer were purchase from Gibco-BRL.

2.3.6 Polymerase Chain Reaction (PCR) Amplification

PCR reactions were set up by combining 33.5 μ l dH₂O, 2.5 μ l DMSO to 5% final concentration, 1 μ l chromosomal or plasmid DNA, 2 μ l primer-up and primer-down, [30 pmoles each], 5 μ l 2 mM dNTP, 5 μ l 10 \times *Taq* buffer, which also contained 1.5 mM Mg²⁺, and 50 μ l of mineral oil overlay to prevent reaction mixture evaporation. The thermal cycles consisted of the following steps: 5 min at 96⁰C denaturing; added 1 μ l thermal stable DNA polymerase, *Taq* Polymerase; 1 min at 95⁰C; 45 s at X_{ave}⁰C annealing temperature, where

$$X_{ave} = (X_{\text{primer-up}} + X_{\text{primer-down}}) / 2$$

$$X_{\text{primer-up/down}} = [4(G + C) + 2(A + T)] - 5;$$

Y min at 72⁰C extension time where Y = 1 min/Kb; 35 cycles from step 2 to step 4; 10 min at 72⁰C, and (7) infinite min at 4⁰C storage step. The volume of dH₂O used at step 1 was relative to the volume of DNA template added at step 3.

In some instances the DNA template was prepared by colony boiling preparation. A single colony was suspended in 50 μ l of dH₂O, centrifuged at 12,000 rpm for 1 min, and resuspended in 50 μ l of dH₂O. The suspension was then boiled for 5 min, followed by another centrifugation cycle. 5 μ l of the DNA containing supernatant was used in the PCR reaction.

2.4 COMPETENT CELL PREPARATIONS

Chemically competent *E. coli* cells were prepared by first picking a single colony of the desired strain from an agar plate, inoculating it in 4 ml of LB, and growing it as described in section 2.2. The O.N. culture was subcultured in 40 ml of LB and grown to log phase, ($OD_{600nm} = 0.6-0.8$). Next, the cells were chilled on ice for 10 min, and pelleted at 4°C, 5000 rpm, for 10 min in a Beckman Model J2 centrifuge. While on ice, the supernatant was decanted, and the pellet was resuspended with 20 ml of ice cold sterile 0.1 M $MgCl_2$ by gentle pipeting. The cells were then held on ice for 15 min. The cells were centrifuge again at the same specifications as above. The supernatant was decanted and the pellet was resuspended with 20 ml of TG-salts, which contains 75 mM $CaCl_2$, 6 mM $MgCl_2$, and 15% glycerol. The cells were held on ice for 30 min. Once again the suspension was pelleted and the supernatant was decanted. 1 ml of ice cold TG-salts was used to resuspend the pellet. The cells were incubated O.N. at 4°C, and were then separated into 1.5 ml microfuge tubes in 200 μ l aliquots. Prior to storage at -70°C, the cells were frozen on dry ice for 5 min.

Electrocompetent *P. aeruginosa* cells were prepared by subculturing an O.N. culture in 100 ml of LB and were allowed to grow to log phase (A_{600nm} 0.5-0.8). They were then placed on ice for 15 min, and pelleted at 6000 rpm, 4°C, for 10 min in pre-chilled centrifuge tubes. The supernatant was decanted and the cells were washed in 5 ml of ice cold 1 mM HEPES (pH 7), and centrifuged as described above. Washing and centrifugation were repeated twice. After the final spin, the cells were resuspended in 1 ml SMEB, which contained 1 mM HEPES (pH 7), 300 mM sucrose, and 1 mM $MgCl_2$. 100 μ l aliquots were pipetted in pre-chilled eppendorf tubes and stored at -70°C.

Turbo-competent cells were prepared in a similar fashion as above with minor exceptions. These cells were prepared when it was not necessary to make a large batch of competent cells for a specific strain. O.N. cultures were transferred to eppendorf tubes at room temperature, and centrifuged at a bench top centrifuge for 30 s, 12,000 rpm. The supernatant was decanted and the cells were suspended in 1 ml of cold 0.1 M MgCl_2 . They were centrifuged again for 30 s at 12,000 rpm. The supernatant was decanted, and the pellet was resuspended with 250 μl of cold TG-salts. All 250 μl of competent cells were used during transformation.

2.5 MECHANISMS OF DNA TRANSFER

2.5.1 Transformation

Transformations were accomplished by first allowing 100-250 μl of the desired competent cells to thaw on ice for ~10 min. 20 μl (ligation reactions) or 5 μl (concentrated plasmid DNA) was pipetted directly into the competent cells and the mixtures were chilled on ice for 30 min. They were then heat shocked for 2 min at 42°C without shaking. Next, the cells were suspended in 1 ml of LB and incubated for one hour in a 37°C shaker. 200 μl aliquots were plated on selective medium, allowed to dry, and incubated upside down for 24 h in a 37°C incubator. The rest, ~800 μl , was centrifuged at 7000 rpm for 1 min, resuspended in 200 μl of supernatant, and plated as described above.

2.5.2 Biparental Mating – Conjugation

O.N. cultures of SM10 *E. coli* cells, which contained the plasmid to be conjugated, and the *P. aeruginosa* strain to receive the plasmid were diluted 1:50 in LB without antibiotic. Each strain was grown to log phase. 700 µl of each were mixed together and centrifuged for 1 min at 7000 rpm. 30 µl of supernatant was used to resuspend the pellet, and was placed on a warm transfer filter that rested in the middle of an agar plate lacking antibiotics. SM10 cells containing pFLP2 were incubated with *P. aeruginosa* strains for 3 h at 37°C. SM10 cells containing other plasmids were incubated at 37°C for 24 h. After incubation, the transfer filter was vortexed in 1 ml of PBS. 200 µl aliquots along with dilutions in PBS up to 10⁻⁵ were subsequently plated on selective medium and incubated for 24 h.

2.5.3 Transduction

Transduction was accomplished as outlined in section 2.6 with DL291 *E. coli* cells. DL291 cells are *recA*⁻ and Δ *lacZ*. It was important to use *recA*⁻ cells to prevent recombination of the phage. Δ *lacZ* cells were obviously important to prevent chromosomally – encoded β -Galactosidase from interfering with the β -Galactosidase assays.

2.6 BACTERIOPHAGE λ

Bacteriophage λ RZ5 (phage λ RZ5) was used as a cloning vector to introduce the *PmexJK-lacZ* fusion system into *E. coli*. The kanamycin resistance gene from pUCK4 was used as a selectable marker. Once integrated at the *attP/attB* sites, the phage is

referred to as a prophage. A lysogen, on the other hand, is the bacterium that contains a prophage (70). Phage λ was used because it integrates as a single copy at the phage *attP* site and bacterial *attB* site, which maintained the integrity of the *PmexJK-lacZ* cloned element. This eliminated artifacts generated when using high copy number cloning plasmids. Phage λ is also stable enough in *E. coli* to be maintained without selection (56). Most importantly, the purpose of cloning the *PmexJK-lacZ* fusion system into *E. coli* was to see if the repressional activity of MexL is influenced by an activator/inducer or corepressor that may be absent in *E. coli*, but present in *P. aeruginosa*.

2.6.1 Viral Plaque Isolation and Spontaneous Excision Frequency

These two methods are one in the same. Lysogens were grown O.N. as described in section 2.2 and the cultures were centrifuged. 100 μ l of chloroform was vortexed with 4 ml of O.N.. 1 ml was transferred to a microfuge tube and centrifuged for 1 min. The supernatant was transferred to a sterile glass vial, which was vortexed briefly with 50 μ l of chloroform. The lysate was stored at 4°C. The lysate was serially diluted in phage dilution buffer described in section 2.2, and spot plated on a top agar containing MC4100 *E. coli* and X-Gal. Top agar was prepared by autoclaving 100 ml of TB broth with 0.7 g of agar. 3 ml aliquots were put into 5 ml transformation tubes where it cooled to 50°C in a block heater. Once at 50°C, 100 μ l of MC4100 cells that were suspended in 10 mM MgSO₄ were pipetted into the top agar. If the top agar was poured on top of LB plates, X-Gal was introduced into the top agar while in the block heater. If the top agar was poured on a MacConkey plates, X-Gal was omitted. The top agar was allowed to dry

prior to incubation. After 24 h of incubation blue plaques (LB plates) and red plaques (MacConkey plates) were counted visually (21).

2.6.2 Transfer of *lacZ* Fusions to Phage λ RZ5

100 μ l of the fusion containing plasmid (pPS1193) in MC4100 suspended in 10 mM MgSO₄ and 100 μ l of a high titer lysate of λ RZ5 were mixed together in a microfuge tube. The mixture was incubated for 10-15 min at 37°C, and after being microfuged to pellet the cells were washed twice with 1 ml of LB. Finally, the pellet was resuspended in 1 ml of LB and incubated for 1 h at 37°C without shaking. 100 μ l aliquots were plated on selective medium (LB_{Km35}, X-Gal).

2.6.3 Preparation of High-Titer Lysates

Agar plugs containing single plaques were pulled out of the top agar using disposable Pasteur pipettes, and placed in 15 ml culture tubes containing 1-2 drops of MC4100 *E. coli* cells. The first culture tube contained no plaques (growth control), the second contained one plaque, the third contained two plaques, and so on through culture tube 5. 2 ml of LB broth was put into each tube with 10 mM MgSO₄ (final concentration), and the tubes were shaken vigorously at 37°C until visible cell lysis occurred between 5-8 h. The tubes were removed from the 37°C shaker, and several drops of chloroform were vortexed into the mixture. The procedure outlined in section 2.6.1 was used to determine which of the five samples had the lowest input phage and the highest viral titer (65).

2.7 PLASMID ELECTROPORATION

Plasmids electroporated into various *P. aeruginosa* strains were eluted with dH₂O (section 2.3.1). 1-10 µl of DNA was pipetted into electrocompetent cells after they were allowed to thaw for ~10 min (section 2.4). The DNA and cells were incubated on ice for 1 min. The mixture was transferred to pre-chilled electroporation cuvettes (0.2 cm gap), and placed in a Bio-Rad Gene Pulser electroporation apparatus. The voltage was set at 2.5 kV, discharge capacitor at 25 µF, and the pulse controller parallel resistor was set at 200 Ω. Immediately after the capacitor discharged, 900 µl of ice cold LB was applied to the cuvette. The mixture was transferred to 5 ml glass transformation tubes, and were incubated on ice for 30 min. Next, the cells were incubated in a 37°C shaker for 1-2 hours, and plated on selective medium.

Plasmids electroporated into *P. aeruginosa* were verified by reisolating them as described in section 2.3.1, and restriction digest as described in section 2.3.3.

2.8 FLP-MEDIATED EXCISION

pFLP2 was conjugated into the appropriate *P. aeruginosa* strains, containing a gentamycin resistance marker (section 2.5.2). The exconjugants were selected on VBMM_{Cb200} (section 2.2). Colonies were test patched on VBMM_{Cb200}, master plate, and VBMM_{Gm15} to test for Gm susceptibility. VBMM_{Cb200}^r and VBMM_{Gm15}^s colonies were plated on VBMM 5% sucrose to cure the pFLP2 plasmid. Resulting colonies were then test patched on a VBMM master plate, VBMM_{Cb200} for susceptibility indicating the absence of pFLP2, and VBMM_{Gm15} for susceptibility indicating the proper excision of the gentamycin cassette (24).

2.9 Allelic Exchange

Plasmid constructs were engineered that utilized *Pseudomonas* suicide cloning vectors, pEX18Ap and mini-CTX3-*lacZ*, that contained the appropriate target gene(s). These constructs were transformed into the plasmid mobilizer strain SM10, and conjugated into the appropriate *P. aeruginosa* strain where homologous recombination occurred between the plasmid vector and host chromosome. The exconjugants, which in some cases represented a mixed population of mutant strains and merodiploids, were selected on VBMM_{Gm15}. Merodiploid strains were eliminated from the mixture by plating on VBMM_{Gm15} 5% sucrose. Finally, pFLP2 mediated excision (section 2.8) was used to excise the gentamycin antibiotic marker from the host chromosome, which generated unmarked mutants or in some instances unmarked *lacZ* fusion strains (60).

2.10 β -GALACTOSIDASE ASSAY

LB cultures were incubated O.N. in a 37⁰C shaker, and were then subcultured 1:100 and grown to log phase in LB. M9/casamino acid cultures were grown O.N. without subculturing. OD_{600nm} were read for each culture just prior to the assay. Assay mixtures contained 50 μ l culture; 450 μ l Z-buffer; 10 μ l chloroform; 10 μ l 0.1% SDS, after vortexing for 10 s; 100 μ l of 4 mg/ml ONPG was added; after sufficient yellow color had developed, the reaction was terminated by adding 250 μ l 1 M Na₂CO₃. The samples were centrifuge at 12,000 rpm for 2 min and the A_{420nm} was read. β -Galactosidase units were calculated using the following formula:

$$\beta = 1000(A_{420nm})/(t)(v)(A_{600nm})$$

Where t is time in minutes at step 7, and where v is volume in ml at step 1. Z-buffer was made by adding 16.1 g $\text{Na}_2\text{HPO}_4 \times 7 \text{ H}_2\text{O}$, 5.5 g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$, 0.75 g KCl, and 0.25 g $\text{MgSO}_4 \times \text{H}_2\text{O}$ to 1 liter H_2O . Z-buffer was not autoclaved and was stored at 4°C . Just prior to use, 270 μl of β -mecaptoethanol per 100 ml of Z-buffer was added. ONPG was dissolved in dH_2O , and stored in a 25 ml culture tube surrounded by tin foil at 4°C . Each strain was assayed in triplicate. A blank for the spectrophotometer lacking bacterial cells was made at the same time as the test samples (46).

2.11 MINIMUM INHIBITORY CONCENTRATION (MIC) TESTS

Microtiter plate MICs were accomplished in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards. Briefly, 50 μl of two fold serial dilutions at twice the desired antibiotic/antiseptic concentration were pipetted into labeled microtiter plate wells. Antibiotic stock concentrations were dissolved in the proper solvent (table 2.4) at 1000 $\mu\text{g/ml}$ or 10 times the highest concentration to be tested which ever was greater. *P. aeruginosa* strains were plated on MH agar and incubated as indicated in section 2.2. Colonies were used within 24 h of incubation. 3-5 colonies were suspended in 2 ml of 0.85% NaCl (saline) and adjusted to the 0.5 McFarland standard, which gave $\sim 1-2 \times 10^8$ CFU/ml. Samples were further diluted in MH broth to 1×10^5 CFU. 50 μl of these cells were inoculated in triplicate into their proper wells using a multipipeter, which reduced the antibiotic concentration to the desired level. Inoculums were used within 15 min of preparation. The microtiter plates were placed in a humidifying chamber, and were read after incubation in a 37°C incubator at 18 and 24 h (48).

E-tests MIC determinations were accomplished in accordance with the AB Biodisk protocol. E-tests were used for ciprofloxacin and tetracycline MIC determinations only. E-test strips were purchased from AB Biodisk (Piscataway, NJ).

2.12 GENOMIC SOUTHERN HYBRIDIZATION

1-1.5 µg of chromosomal DNA was digested with *Nco*I for 4 h at 37°C. The fragments were electrophoresed on a 1% TAE agarose gel as described in section 2.3.3. 1 µl of prebiotynlated marker with 17 µl of dH₂O, and 2 µl of REact 3 was ran in place of the Hi-Lo DNA marker. The DNA was transferred to a Photogene nylon membrane (Gibco-BRL) as described by Sambrook et al. (58).

PCR amplification and the *mexL*-up / *mexL*-down primers were used to obtain a *mexL* probe which was biotinylated using the NEBlot™ Phototype™ kit from New England Biolabs (Beverly, MA). A gentamycin resistance cassette was obtained by restriction digest of pPS856 and the fragment was gel purified (sections 2.3.3 and 2.3.4) from plasmid pPS856. It was biotinylated in the same fashion as the *mexL* probe. The Photogene nylon membrane was probed with biotinylated markers in accordance with the Phototype™ detection kit protocol. Hybridization was accomplished at 68 °C for 24 h with prehybridization fluid, which contained a final concentration of 6 x SSC, 5 x Denhardt's Reagent, 0.5% SDS, and 100 µg/ml of denatured salmon sperm DNA. Hybridized fragments were detected by exposure of the membrane to FUJI X-ray film.

CHAPTER 3

RESULTS

3.1 ISOLATION OF A *mexL* DELETION MUTANT

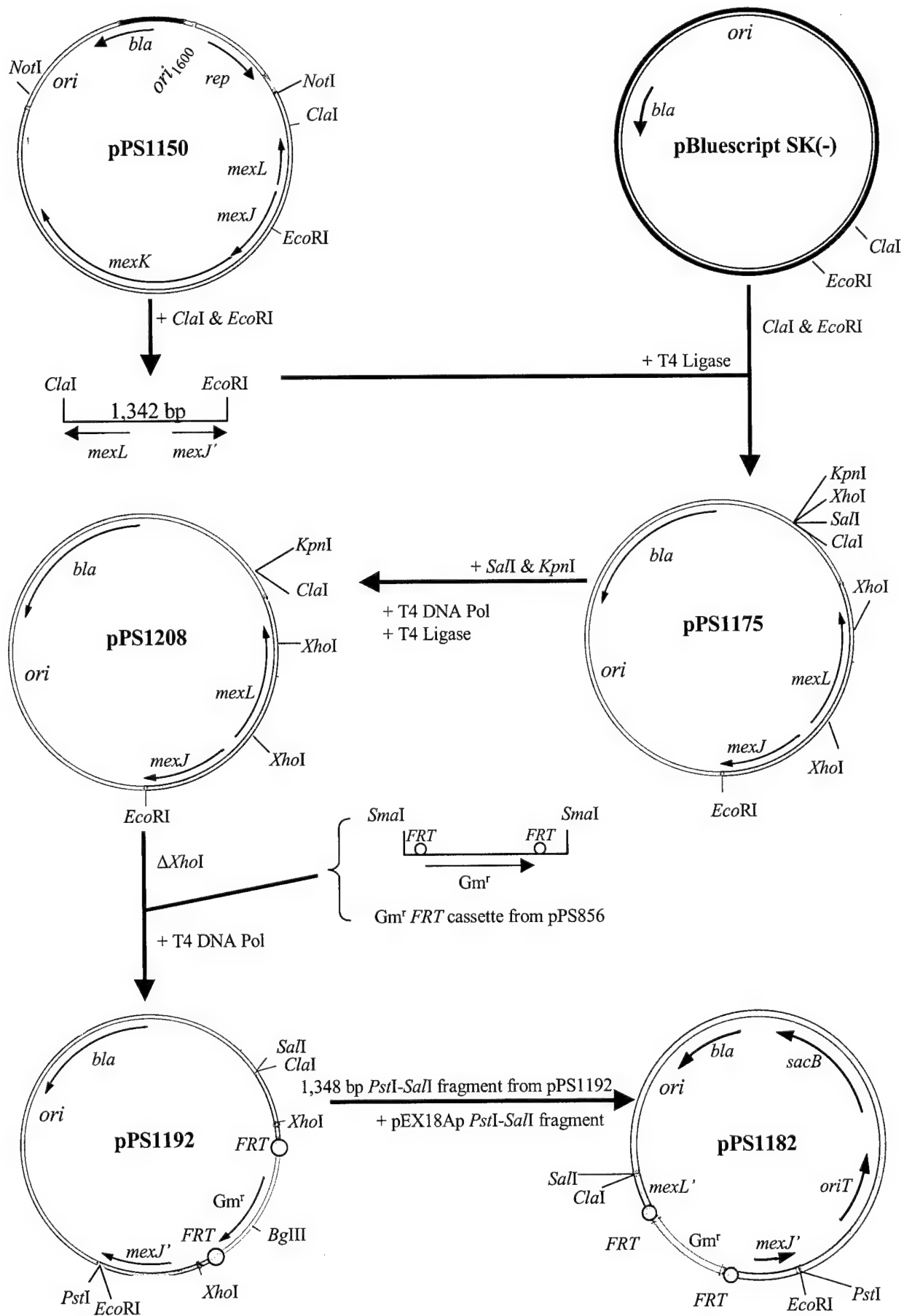
PAO238-1 was previously isolated as a spontaneous triclosan resistant mutant, and was shown to contain a point mutation in the putative regulatory gene *mexL* (12). PAO238-1 was highly triclosan resistant, and showed a MIC >128 µg/ml. When PAO238-1 was complemented with *mexL* via pPS1153, the MIC was lowered to 20 µg/ml. This strongly suggested that MexL was a negative regulator of the *mexJK* operon. However, it was possible that other genes of the PAO238 chromosome were either mutated or activated in a fashion that may explain the expression of *mexJK*. Hence, one goal of this study was to engineer a *mexL* mutant strain in the parent, PAO238, and characterize it. These data were compared to the results previously obtained from the PAO238-1 experiments.

We hypothesized that if MexL is a negative regulator of the *mexJK* operon, then we should observe the same or greater levels of triclosan resistance in the newly constructed *mexL* mutant strains as in PAO238-1. Likewise, we expected to see the same decrease in triclosan resistance of a Δ *mexL* mutant when complemented with *mexL* via pPS1153.

3.1.1 Subcloning of the *mexL* Gene

The plasmid pPS1150, containing a 6.6 kb region of the *P. aeruginosa* chromosome including *mexLJK*, was transformed and propagated in *E. coli* JM110. JM110 is a DNA deoxyadenosine methylase (*dam*) mutant. The *Cla*I site on pPS1150 was Dam methylated, which made it immune to *Cla*I digestion. Dam methylates all adenines at the N⁶ position with 5'-GATC-3' sequences. Methylation is critical in methyl-directed mismatch repair. It allows cellular enzymes to distinguish between the template strand and newly synthesized stand of DNA from its degree of methylation (76). By propagating pPS1150 in JM110, the *Cla*I site became available for digest due to its lack of methylation.

After isolation of pPS1150 DNA from JM110, a 1,342 bp *Cla*I-*Eco*RI fragment that contained *mexL*, the *mexL-mexJ* intergenic region, and 472 bp of *mexJ* from pPS1150 was cloned into pBluescript (plasmid constructions are illustrated in figure 3.1). This *mexL* fragment disrupted the *lacZ* gene coding for β -Galactosidase in pBluescript. Blue/white screening was used to select for the newly constructed plasmid pPS1175, which was plated on LB_{Ap100}, X-Gal. *E. coli* DH5 α F' strains that contained just the pBluescript vector yielded blue colonies indicating the functionality of its *lacZ* gene, which ultimately indicated the failure of *mexL* subcloning. These colonies were obviously ignored. White colonies, on the other hand, indicated that the insertion of *mexL* in pBluescript was successful. These colonies were picked and verified using restriction digests and resulted in the isolation of plasmid pPS1175.



3.1.2 Engineering an Unmarked Chromosomal *mexL* Deletion Mutant

pPS1175 was double digested with *SalI* and *KpnI* to eliminate their intervening *XhoI* restriction site. Once this *XhoI* site was removed, the remaining ends were blunt end ligated, which generated another *KpnI* site. A *KpnI* digest was accomplished on this construct, pPS1208, to verify its integrity. Next, a 372 bp fragment was removed from *mexL* via *XhoI* restriction digest. The *XhoI* sites of this fragment were blunt ended, and ligated to a gentamycin resistance (Gm^r) cassette flanked by two *FRT* sites from the plasmid pPS856. This new constructed was designated pPS1192.

A 1,348 bp *PstI-SalI* fragment that contained the mutated *mexL::Gm^r-FRT* region from pPS1192 was cloned between the same sites of pEX18Ap, a cloning suicide vector, to yield pPS1182. This plasmid contained the counter-selectable *sacB* gene to force subsequent double recombination events. It also contained a *bla* ampicillin (or carbenicillin in PAO) resistance gene for selection of pPS1182 receiving strains, and detection of merodiploids once integrated into the host chromosome. This plasmid was amplified in DH5 α F', isolated, and transformed into the plasmid mobilizing *E. coli* strain SM10. From this strain, pPS1182 was conjugated into PAO238 where double reciprocal homologous recombination occurred between wt *mexL* on the PAO238 chromosome and the mutated *mexL* region on pPS1182 (figure 3.2). This new strain was named PAO319. Next, Flp-mediated excision was performed to remove the Gm^r marker from PAO319, which generated the "unmarked" *mexL* mutant, PAO318 (figure 3.3).

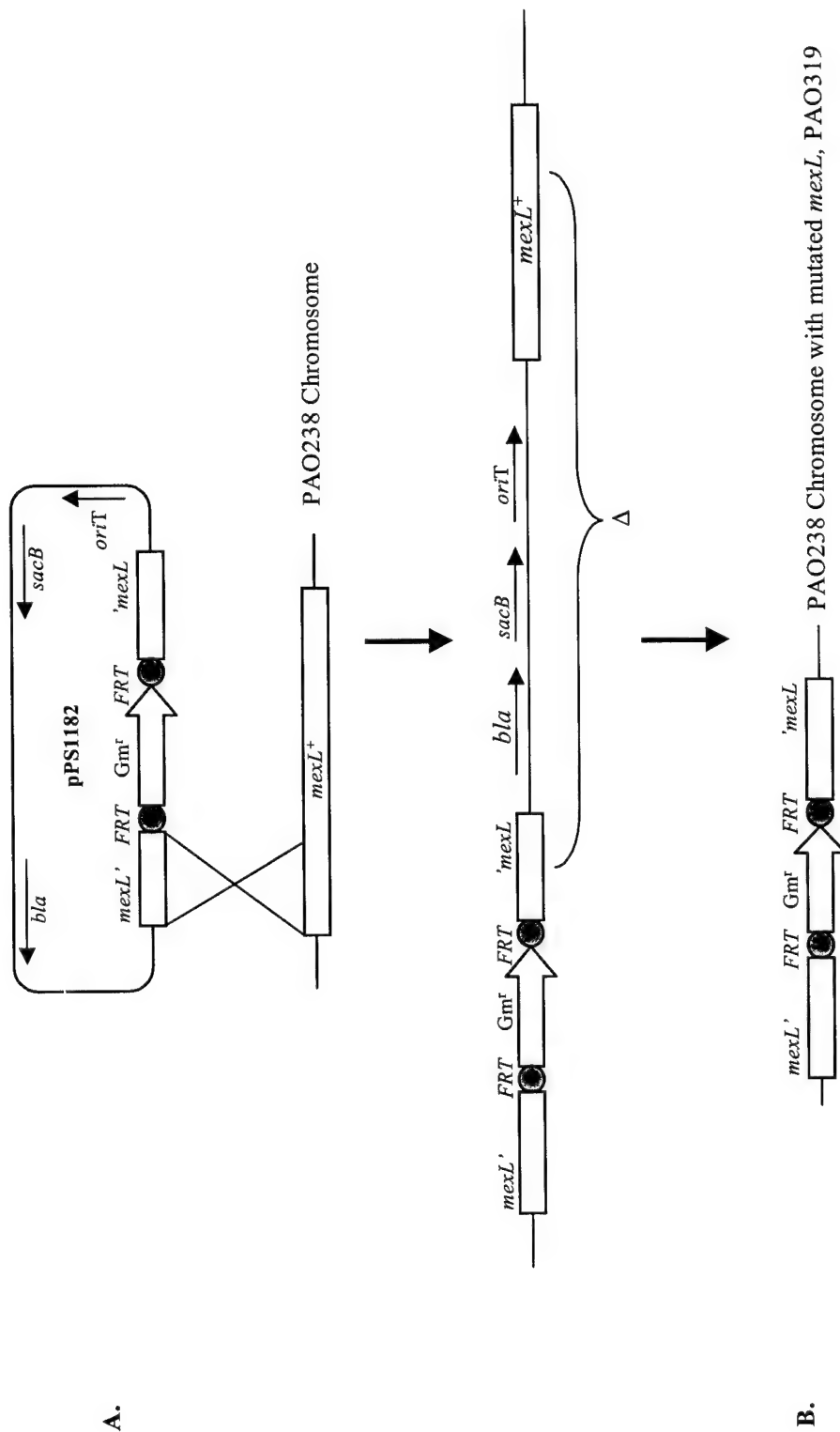


Figure 3.2. Construction of a Chromosomal $\Delta mexL$ Mutation. **A.** Single crossover recombination event that resulted in a merodiploid. Note that the entire pPS1182 plasmid has integrated into the PAO238 chromosome. **B.** This depicts the second recombination event which occurred when the merodiploid was plated on VBMM 5% sucrose in the presence of gentamycin. The *sacB* gene located on the backbone of pPS1182 forces the second event by coding for a protein that in the presence of sucrose is cytotoxic to the cell. The result is the generation of a Gm^r-marked *mexL* mutant, and the deleted sequences are lost. This strain was named PAO319.

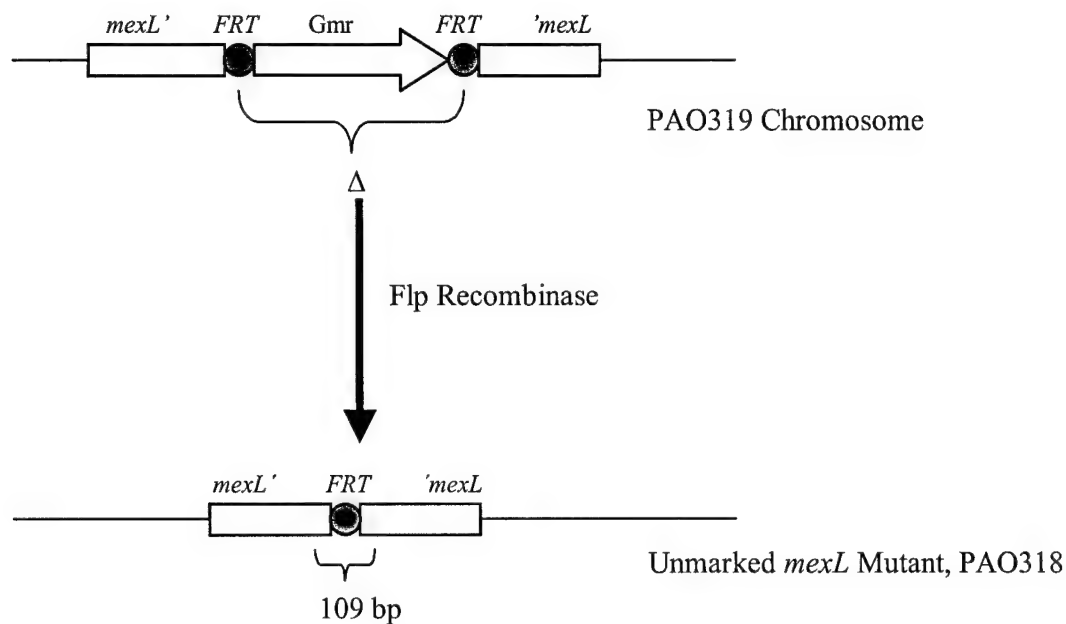


Figure 3.3. Flp-Mediated Excision of the *Gm^r* Cassette. SM10 containing the pFLP2 plasmid was conjugated into PAO319. Flp recombinase encoded by this plasmid recognized the *FRT* sites in the PAO319 chromosome and excised the *Gm^r* cassette. Since pFLP2 contained a *sacB* gene, these mutants were plated on VBMM 5% sucrose to cure the plasmid. This resulted in the unmarked *mexL* mutant, PAO318.

3.1.3 Minimum Inhibitory Concentration (MIC) Tests

The MICs of PAO318 and PAO319 for various drugs can be found in figure 3.4 and table 3.1. The triclosan MICs of PAO318 and PAO319 were both >128 µg/ml. The unmarked mutant, PAO318, was useful in that it did not interfere with gentamycin MIC determinations.

PAO318 was complemented with *mexL* by electroporating pPS1153 into electrocompetent PAO318 cells. The vector, pUCP20T, the plasmid backbone of pPS1153, was used as a vector control in this experiment. In the pPS1153 transformants, the MIC for triclosan fell to 20 µg/ml, which is the same MIC previously observed for PAO238-1 when it was complemented with pPS1153.

PAO1, PAO238, and PAO238-1 were all control strains that were assayed. PAO1, constitutively expressed MexAB-OprM and was highly resistant to triclosan. In contrast, PAO238, a $\Delta(mexAB-oprM) \Delta(mexCD-oprJ)$ double efflux pump deletion mutant, was highly sensitive to triclosan. PAO238-1, containing a *mexL* point mutation and therefore constitutively express *mexJK*, was resistant to triclosan. PAO318 and PAO319, the $\Delta mexL$ strains, were highly resistant to triclosan. PAO318 with pUCP20T was the vector control for the complementation experiment. Its MIC was unaffected by the presence of pUCP20T. PAO318 with pPS1153 showed the same MIC as PAO238, 20 µg/ml.

MICs were also determined for several antibiotics (table 3.1). The results are consistent with the data previously obtained from PAO238 and PAO238-1. The MICs for these antibiotics do not change significantly for PAO318 and PAO319 since these

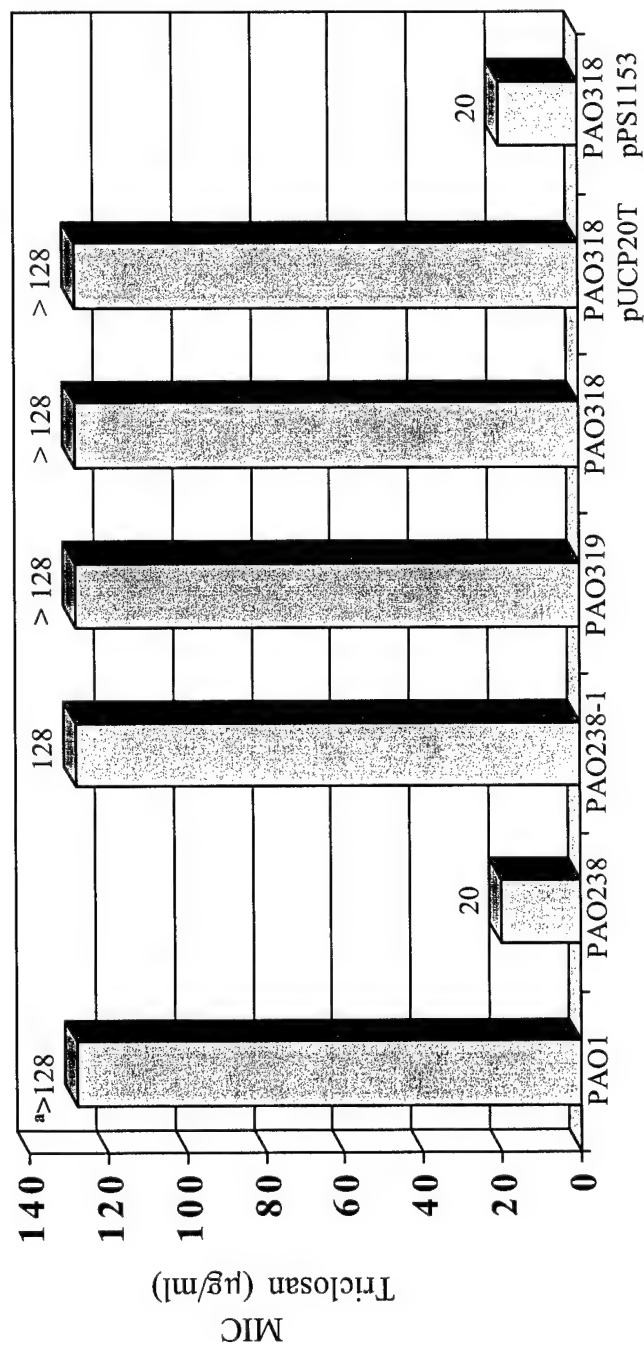


Figure 3.4. Microtiter Plate Minimum Inhibitory Concentration (MIC) for Triclosan of a $\Delta mexL$ Mutant. Each bar represents a unique *P. aeruginosa* strain. ^a The numbers above the bars indicate the actual MIC values in $\mu\text{g/ml}$.

Table 3.1
Minimum Inhibitory Concentrations (µg/ml)

Strain (Plasmid)	Efflux Pump Expressed	Tri ^a	Bis	Hch	Acr	Tc	Cip	Tmp	Gm	Cb
PAO1	MexAB-OprM	4	>128	16	.064	512	1.2	32	512	1024
PAO238	None	4	32	.75	.006	32	.2	1	32	32
PAO238-1	MexJK	8	32	1	.004	16	.4	1	16	64
PAO238-1 (pPS1153)	None	8	16	0.75	0.006	8	0.2	ND ^c	16	64
PAO 318	MexJK	16	8	.75	.006	32	.125	.05	16	256
PAO318 (pUCP20T)	MexJK	16	8	.75	.006	32	.125	ND	16	128
PAO318 (pPS1153)	None	4	32	1	.004	16	.4	ND	32	32

^a For abbreviations see List of Abbreviations

^b At concentrations >128 µg/ml, triclosan is insoluble in aqueous solutions

^c Not done, since the plasmids coded for a β-lactamase

mutants are lacking an outer membrane protein channel that is required for antibiotic efflux but not for the efflux of triclosan (12).

3.1.4 Genomic Southern Hybridization

Southern blot analysis using a biotinylated gentamycin probe from the plasmid pPS856 demonstrated that the Gm^r cassette was present in PAO319 (figure 3.5). Furthermore, it was properly excised by Flp recombinase in PAO318, as indicated by the absence of a biotinylated band. When compared to the controls PAO1 and PAO238, Southern blots of PAO319 using a *mexL* probe revealed the expected upward band shift from 2,276 bp to 2,982 bp. However, Southern blot analysis failed to demonstrate the expected downward band shift from 2,276 bp to 2,005 bp in DNA from PAO318 showing the net loss of *mexL* in the PAO318 chromosome.

Using the *mexL*-up and *mexL*-down primers (table 2.3), PCR amplification was accomplished on chromosomal DNA from PAO318 and PAO319 in an effort to visualize the partial deletion of *mexL*. All PCR products from PAO319, but not PAO318 yielded DNA bands of the correct size (data not shown). It was then realized that the genome of PAO318 contained 3 independent *FRT* sites. Two of these sites were the result of the deletion mutations engineered to construct the parental strains, PAO200 and PAO238. The third site was left behind when excising the Gm^r cassette from PAO319. Each of these three sites had the potential to be recognized by Flp recombinase during excision of the Gm^r cassette of PAO319. It has been shown by Barekzi et al. (4) that large DNA rearrangements due to Flp recombinase activity are possible, and yield no net gain or loss of DNA.

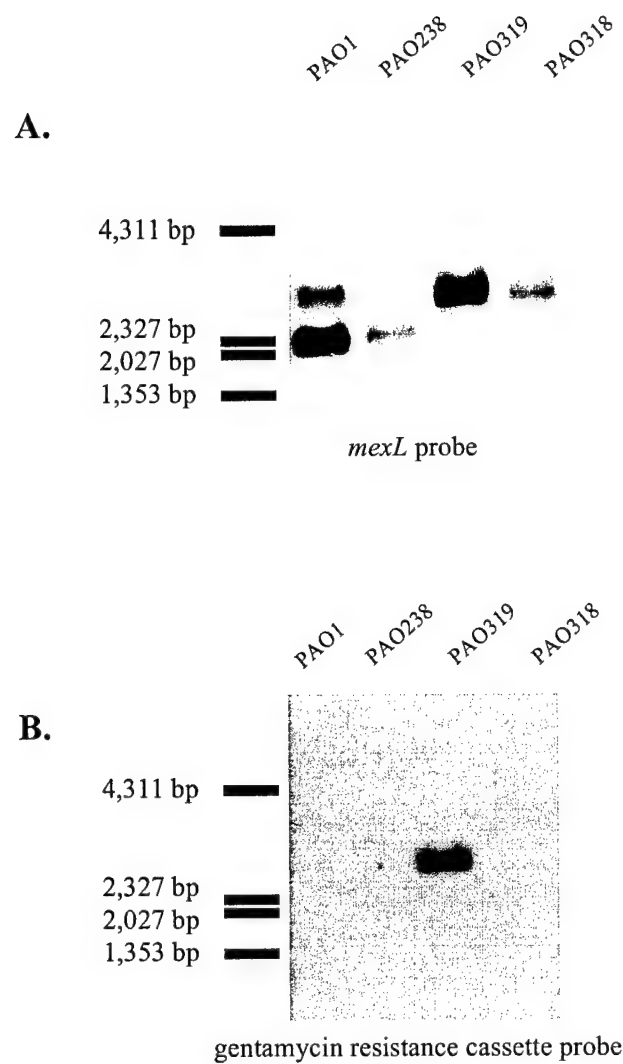


Figure 3.5. Southern Blot Analysis of *mexL* Mutants of *Pseudomonas*. Chromosomal DNAs (1.5 µg/ml) of the indicated mutants were digested with *Nco*I. The fragments were separated by agarose gel electrophoresis and transferred to nylon membranes. The membranes were probed with a biotinylated *mexL* fragment (A.), and a biotinylated Gm^r fragment (B.). The values shown on the left indicate the positions of biotinylated molecular size makers.

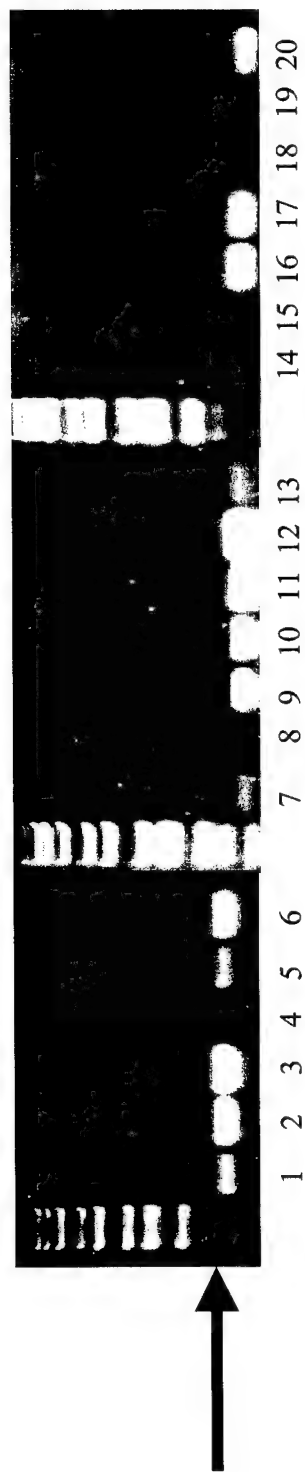


Figure 3.6. PCR Analysis of the *mexL* region After Flp Excision. PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. Lanes 1-3, 5, 6, 7, 9-14, 16, 17, and 20 yielded a *mexL* product at ~ 600 bp. The arrow points to the position of the expected 612 bp fragment. Molecular size markers (top to bottom) were 500 bp, 750 bp, 1000 bp, 1400 bp, 1550 bp, 2000 bp, 3000 bp, 4000 bp, 6000 bp, 8000 bp, 10,000 bp.

To test this hypothesis, Flp-mediated excision was reaccomplished on PAO319. This time, twenty colonies were selected at random after the curing of pFLP2 on VBMM with 5% sucrose. Colony boiling preparations were used as a source for chromosomal DNA templates for PCR amplification. Fifteen of the twenty colonies picked yielded a *mexL* band of the correct size (figure 3.6). However, 5 colonies did not show any DNA fragment suggesting that their chromosome underwent additional rearrangements. It is therefore likely that PAO318 also underwent additional rearrangements during Gm^r cassette excision. Such rearrangements would explain the Southern blot results, and lack of correct PCR products in PAO318. Efforts are currently underway to determine exactly between which *FRT* sites the rearrangement(s) may have occurred.

3.2 REGULATORY STUDIES

In order to quantitate the transcriptional repression activity of MexL on the *mexJK* operon, *lacZ* fusions were engineered by cloning the promoter of *mexJK* in front of a promoterless *lacZ* gene. The reporter gene, *lacZ*, was also used to measure the regulatory activity of MexL in *E. coli*.

3.2.1 Cloning of the *PmexJK* regulatory region into pTZ110

A *lacZ* fusion experiment was accomplished by cloning a *Bam*HI - *Xho*I fragment that contained *PmexJK* from pPS1176 into pTZ110 to give pPS1181 (figure 3.7). This plasmid and pTZ110 were electroporated into PAO238 and PAO318. β -Galactosidase assays were accomplished for all four strains in triplicate per assay. The

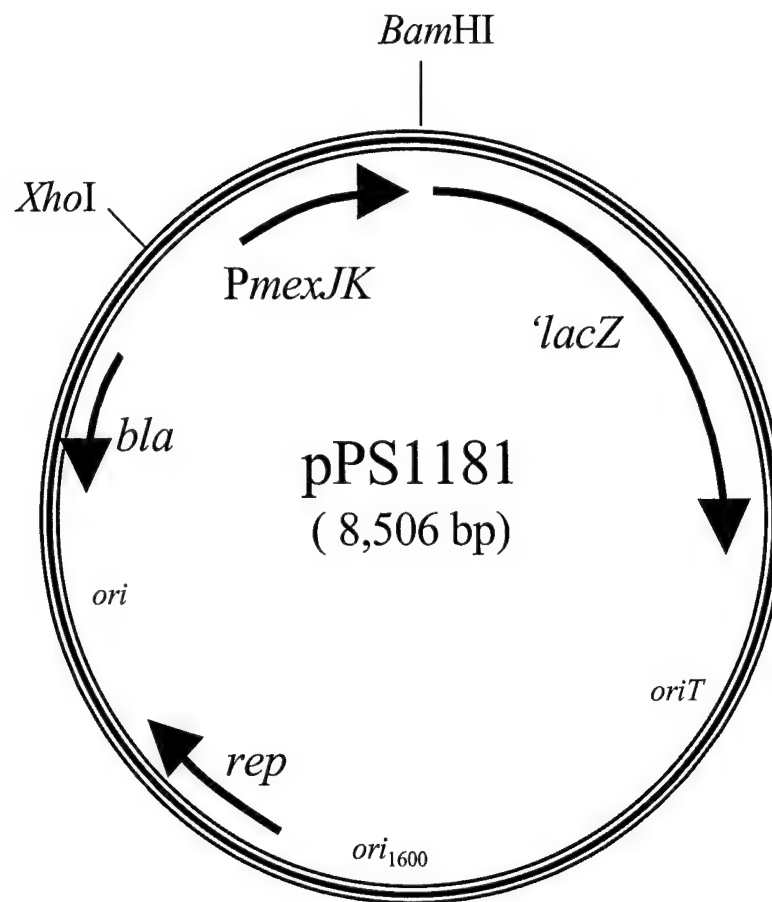


Figure 3.7. Plasmid Map of pPS1181. The promoter of the *mexJK* efflux operon was cloned in front of a promoterless *lacZ* gene in pTZ110 on a 655 bp *XhoI* - *BamHI* DNA fragment from pPS1176. Other abbreviations: *ori*, *E. coli* origin of replication; *ori*₁₆₀₀, broad-host-range origin of replication; *rep*, replication protein required for *ori*₁₆₀₀; *oriT*, origin of transfer; *bla*, β -lactamase encoding gene.

data presented in figure 3.8 represent two independent β -Galactosidase assays at 95% confidence using the Student T Distribution (73):

$$t = \frac{(x - \mu) \sqrt{n}}{s}$$

Where t is Student T Distribution at 95% confidence and n-1 degrees of freedom, X is the mean score in the samples, μ is the mean of all of the scores in the population, s is the standard deviation of the sample, and n is the number of scores in the population.

β -Galactosidase activity were expressed from the *mexJ-lacZ* fusion plasmid, pPS1181, which were contained in the $\Delta mexL$ strain PAO318. β -gal expression from the same plasmid was completely repressed in the *mexL*⁺ strain, PAO238. The levels of β -gal activity observed in this strain were similar to those observed with vector pTZ110 in strains PAO238 and PAO318.

These data indicated that MexL represses *mexJ-lacZ* transcription approximately 15 fold. Since, pTZ110 is a medium copy number (~10-20 copies/cell) plasmid, expression of *lacZ* under *PmexJK* control may not accurately reflect the natural events which occur when regulated in single copy on the chromosome (25). Furthermore, DNA regulation is largely dependent on the nature of DNA supercoiling. Since plasmid and chromosomal DNA supercoiling is different, the plasmid born fusions may not represent the true nature of MexL regulation (16).

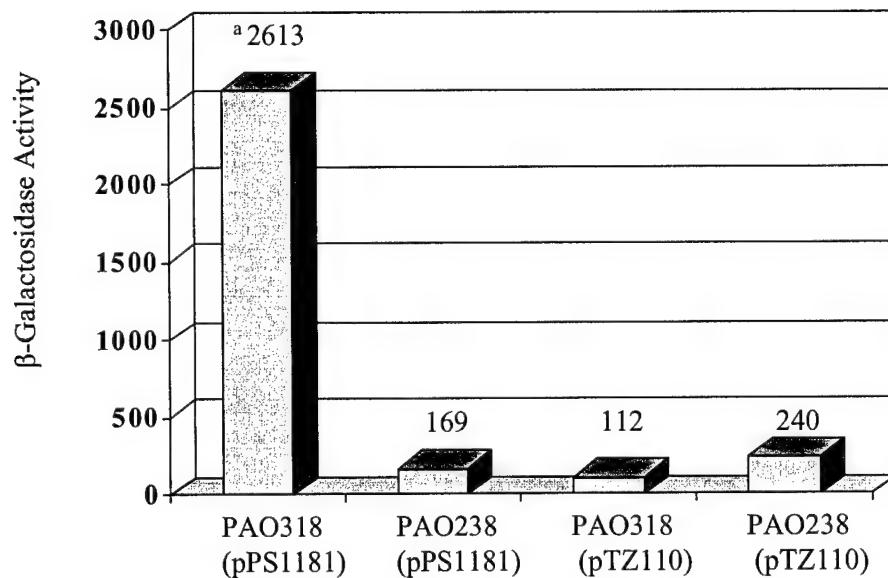


Figure 3.8. Transcriptional Repression of a *mexJ-lacZ* Fusion Contained on pPS1181. β -Galactosidase activity is expressed as $1000(A_{600nm})/(\text{sec})(\text{ml})(A_{420nm})$. PAO238 is a *mexL*⁺ strain and PAO318 is a Δ *mexL* derivative of PAO238. Plasmid pTZ110 is the vector control. Cells were grown in LB-carbenicillin medium to log phase, and β -Gal activities were measured. The values shown are the averages of triplicate measurements meeting 95% confidence limits. ^a The numbers above the bars indicate the actual β -Gal units.

3.2.2 Isolation of Single Copy, Chromosomal *mexJ-lacZ* Fusions

The *PmexJK – lacZ* fusion element was introduced into the PAO314 chromosome in single copy at the *attB* locus. PAO314 is a $\Delta(mexJKL)$ knockout mutant engineered by Chuanchuen et al. (12) from the parent strain, PAO238. PAO314 was specifically chosen for this experiment for reasons that will become evident. Three strains were obtained for this aspect of the study, PAO320, PAO321, and PAO323 (table 2.1).

The integration plasmid pPS1177 was constructed by cloning *PmexJK* in front of the promoterless *lacZ* gene of mini-CTX3-*lacZ* by subcloning a *XhoI-PstI* fragment from pPS1176 (figure 3.9). Initially, this construct was conjugated into PAO238. The *XhoI-PstI* fragment contained 73 bp of *mexL*, which was shown via genomic Southern analysis to have recombined at the wt *mexL* locus of PAO238 rather than at the *attB* locus. Since PAO314 lacked this portion of *mexL*, the only place for pPS1177 to integrate was at the *attB* site. Hence, PAO314 was the choice strain to use for this experiment.

A second integration plasmid, pPS1179, was constructed by cloning a 1,348 bp *SalI-PstI* fragment from pPS1150, that contained the PAO238-1 mutant *mexL*, into mini-CTX3-*lacZ*. The resulting plasmid, pPS1179, was used to generate PAO320. PAO320 was initially constructed so that it could be compared to a similar strain that contained an unmutated *mexL* gene. The goal was to measure repression by MexL as it normally occurred in PAO238 and PAO238-1. In other words, MexL repressional activity in the correct copy number, and *in cis* on the chromosome versus *in trans* on a plasmid vector. Unfortunately, difficulty in cloning the *mexL*⁺ fragment, PAO322, lead to its

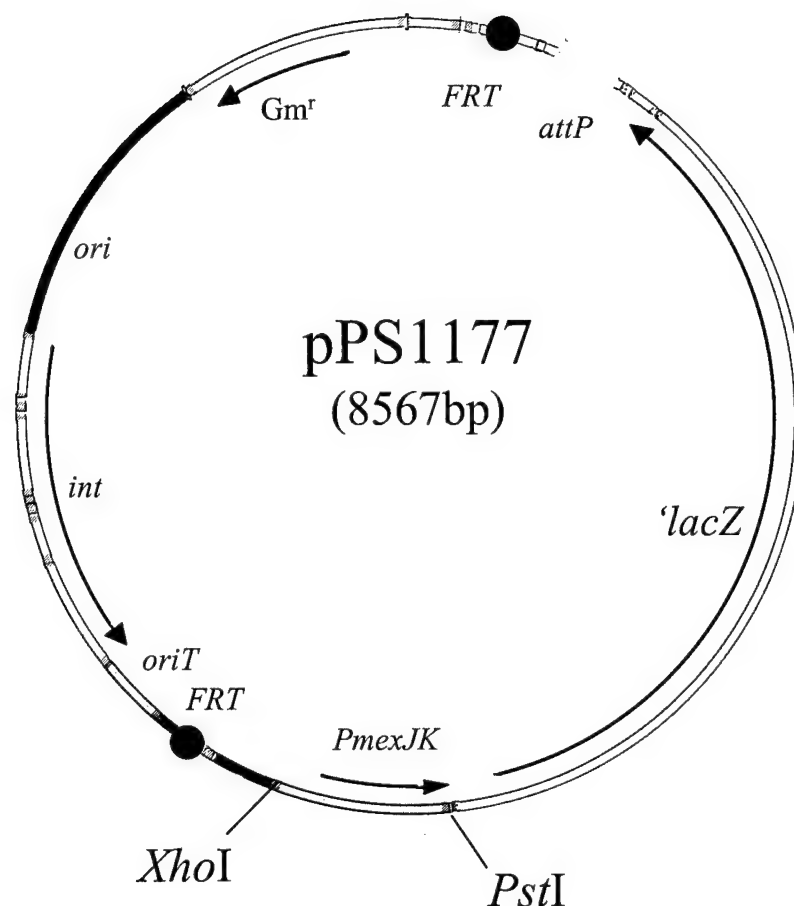


Figure 3.9. Plasmid Map of the Integration Vector pPS1177. This plasmid was designed to introduce fusion elements in single copy at the *attB* locus of the *P. aeruginosa* chromosome. The *attP* site and interase gene whose product drives the recombination at the *attP/attB* sites were from bacteriophage Φ CTX. It also contained an *oriT*, but not an *ori* for replication in *P. aeruginosa*. Hence, it is a suicide vector. The circles represent *FRT* sites that allowed for the Flp mediated excision of the plasmid backbone once it was integrated into the host chromosome (62). pPS1177 was created by cloning the *PmexJK* element in front of the promoterless *lacZ* gene as shown above. The integration vector pPS1179 is similar to pPS1177, but contains upstream sequences with the mutated *mexL* from PAO238-1.

unavailability for a comparative study. However, the data obtained from PAO320 are interesting, and will be discussed in Chapter 4.

To transfer the fusions into the chromosome, mini-CTX3-*lacZ*, pPS1177, and pPS1179 were conjugated into PAO314 and exconjugants were selected on VBMM_{Gm15} agar plates. Homologous recombination occurred at the *attB* locus of each strain to give PAO323, PAO320, and PAO321 respectively. Flp-mediated excision was performed to eliminate the mini-CTX3 backbone, which generated the unmarked strains PAO323, PAO320, and PAO321. PAO323 was the control strain that lacked the *PmexJK* fragment. PAO323 therefore, only contained *lacZ* from the mini-CTX3-*lacZ* plasmid (figure 3.10).

To assess regulation of *mexJ-lacZ* expression, pUCP20T and pPS1153 were transformed into turbo-competent PAO320 cells. Just prior to β -Galactosidase assays, plasmid purification, digestion, and agarose gel electrophoreses were performed on a sample of each to ensure the presence of the correct plasmid. After growth of the cells in LB or M9 medium, β -Galactosidase assays were accomplished twice in triplicate on each of these strains (figure 3.11).

The results obtained showed that in M9 minimal media, supplemented with 0.2% casamino acid, MexL repressed *mexJK* efflux operon transcription approximately 4 fold. However, in LB grown cells, MexL repression was only approximately 1.5 fold.

3.2.3 Isolation of a *mexJ-lacZ* Transducing λ Phage

The *PmexJK-lacZ* fusion was introduced to *E. coli* via bacteriophage λ transduction to evaluate the possibility of effectors that may influence the regulation of *mexJK* operon expression.

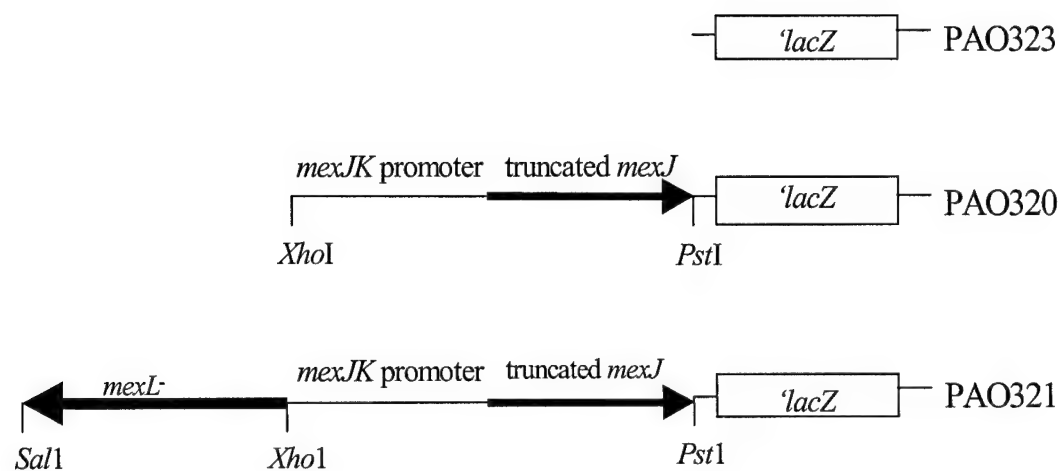


Figure 3.10. *P. aeruginosa* Strains Containing the Indicated Chromosomally Integrated DNA Sequences.

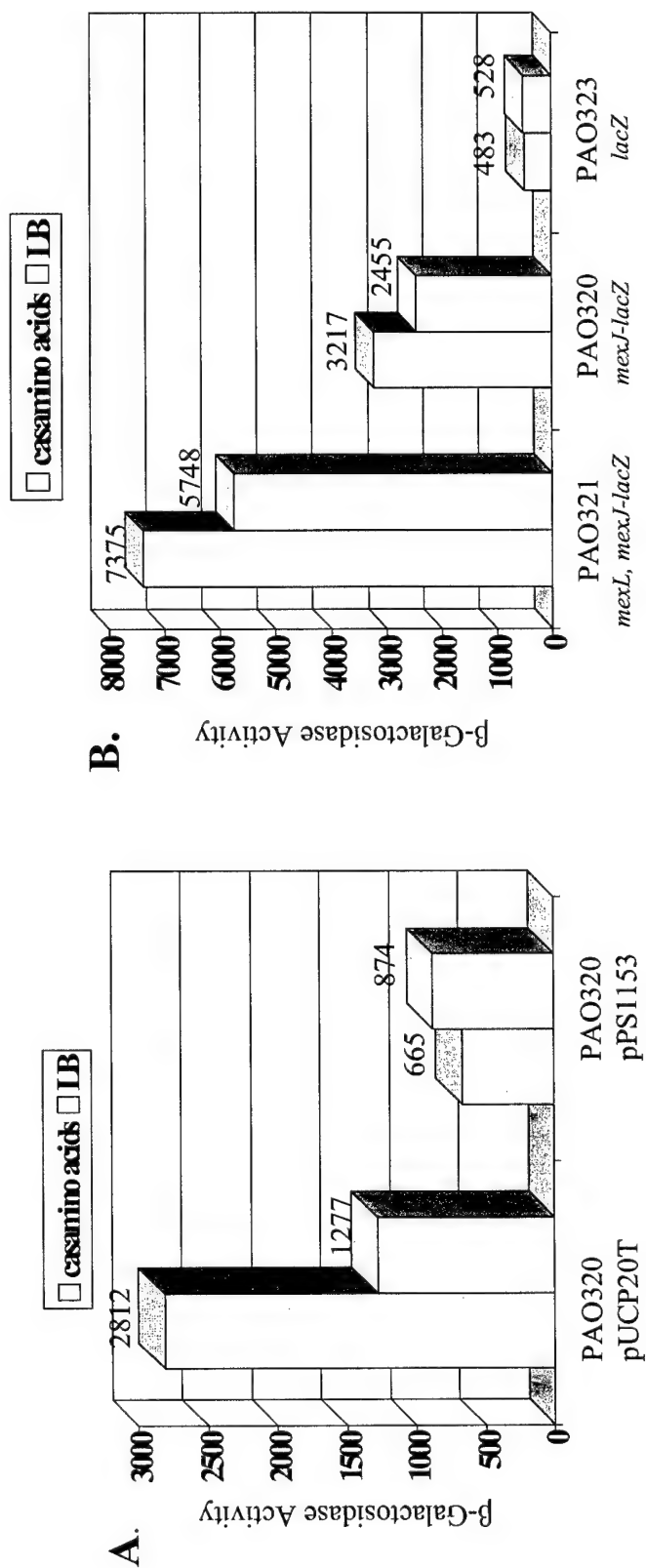


Figure 3.11. β -Gal Expression of Chromosomally Integrated *mexJ-lacZ* Fusions. **A.** PAO320 was complemented with *mexL* via pPS1153. **B.** PAO320, PAO321, and PAO323 were assayed without complementation. Cells were either grown in M9 medium, 0.2% casamino acids, or in LB medium prior to β -Gal assays. Cultures of cells harboring plasmids were supplemented with 100 μ g/ml carbenicillin. β -Gal activities are expressed as $1000(A_{600nm})/(s)(ml)(A_{420nm})$.

To achieve transfer of the *mexJ-lacZ* fusion on λ , a source plasmid, pPS1193, was constructed in three simple steps: First, a kanamycin resistance (Km^r) cassette from pUC4K was obtained after *HincII* digestion and cloned via blunt end ligation into pUC19 that was digested with *ScaI*; this step inactivated the *bla* gene and introduced the Km^r marker; second the correct orientation of the Km^r cassette in pUC19 was verified by digesting DNA from 10 transformants with *XhoI-EcoRI*; if the Km^r cassette was in the desired direction, two bands were visualized on a 0.8% TAE agarose gel at approximately 1,300 bp and 2,700 bp; in the alternative, two 2,000 bp bands would be visualized indicating the undesired orientation of the Km^r cassette. Six of these constructs had the Km^r cassette in the desired orientation; this plasmid was designated pPS1206; and finally, a *KpnI-XbaI* fragment from pPS1179 that contained the *mexJ-lacZ* fusion was cloned into pPS1206 to obtain pPS1193.

For transfer of the Km^r gene and the *mexJ-lacZ* fusion onto λ , pPS1193 was transformed into MC4100 cells, which were $\Delta lacZ$ and *recA*⁺. MC4100 was chosen to transfer the fusion into λ RZ5 because it was λ phage susceptible via an outer membrane receptor, and its RecA protein allowed for the recombination of pPS1193 and the phage genome (figure 3.12). The fusion containing phage was isolated, and high titer lysates were prepared.

DL291 *E. coli* cells were lysogenized with the fusion containing phage. Like MC4100, these cells were $\Delta lacZ$ and λ phage susceptible. However, DL291 was *recA*⁻, which precluded the possibility of further unwanted recombinational events of the transducing phage. The DL291 fusion containing lysogen was designated E1830 or DL291 (λ Mex).

The spontaneous excision frequency of λ Mex was calculated to ensure that phage integration occurred at the *attB* locus. The spontaneous excision frequency was 1.7×10^3 , which suggested that the phage did in fact integrate at the *attB* site and was not “locked-in” at secondary *att* sites (21). β -Galactosidase assays were accomplished to ensure that each λ Mex cell was infected with only one phage. The β -Galactosidase units ranged from 257-266 units, which suggested that only one phage integrated per cell. On the other hand, if double or triple lysogens infected a single DL291 cell, then the total β -Galactosidase activity would have been doubled and tripled, respectively.

The plasmids pUCP20T and pPS1153 were transformed into competent DL291 (λ Mex) cells. β -Galactosidase activity was determined on each strain in a variety of media (figure 3.13). In M9-casamino acid medium, MexL repressed β -Gal expression approximately 4 fold. In LB-grown cells, repression was less than 2 fold.

A.

Growth Medium	Strains		
	λ Mex	λ Mex pUCP20T	λ Mex pPS1153
M9 Glucose	137 u	216 u	38 u
M9 CAA	211 u	214 u	53 u
M9 Glucose & CAA	134 u	188 u	26 u
LB	232 u	181 u	103 u

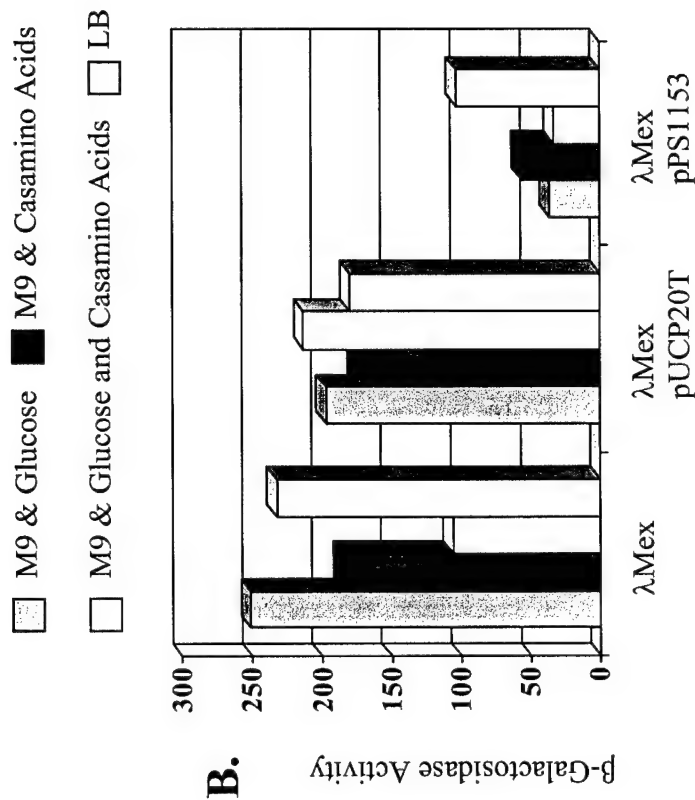


Figure 3.13. β -Galactosidase Assays of *E. coli* λ Mex Lysogens Containing a *mexJ-lacZ* Fusion. **A.** β -Galactosidase units with their corresponding strains and growth media. **B.** Graphic depiction of A. Plasmid pPS1153 expressed *mexL* from the *lac* promoter of the pUCP20T vector. Plasmids were maintained by supplementing the cultures with 100 μ g/ml of ampicillin.

CHAPTER 4

DISCUSSION

The MICs for triclosan obtained with the *mexL* deletion mutant, PAO318, and its complementation with a *mexL* encoding plasmid, pPS1153, supported the hypothesis that MexL is a repressor of the *mexJK* efflux operon. The high ($> 128 \mu\text{g/ml}$) triclosan MIC of PAO318 demonstrated that the absence of MexL repression at the *mexJK* promoter region allowed for the constitutive expression of the MexJK efflux pump. In this situation MexJK effluxed triclosan from the cytoplasm of the cell before it could have a cytotoxic effect on FabI of the fatty acid biosynthesis cycle. Furthermore, wild-type *mexL* that was expressed *in trans* via plasmid pPS1153 generated a functional protein that blocked transcription of the *mexJK* operon. As a result, complemented PAO318 was sensitive to triclosan (MIC $\sim 20 \mu\text{g/ml}$).

PAO318 had multiple *FRT* sites in its chromosome. Two *FRT* sites were generated when *mexAB-oprM* and *mexCD-oprJ* were deleted (12). The third *FRT* site was generated during the excision of the gentamycin resistance cassette from the PAO319 chromosome. Genomic Southern analysis failed to reveal the expected net loss (~ 300 bp) from the *mexL* region of the PAO318 chromosome. Since PCR demonstrated the presence of a *mexL* fragment in fifteen of the twenty subsequent PAO318 strains

cured of the plasmid pFLP2 after gentamycin resistance cassette excision, I hypothesize that multiple rearrangements may have occurred between preexisting and newly generated *FRT* sites during Flp-mediated excision. I further hypothesize that these rearrangements probably generated inversions rather than deletions, since large deletions would likely be deleterious to the organism. After engineering unmarked mutants using Flp-mediated excision (4, 24, 60), PCR amplification should be accomplished using flanking *FRT* site primers to ensure that only the excision event occurred rather than inversions.

Regulatory studies using chromosomal *mexJ-lacZ* fusions have shown that when cells are grown in M9 minimal media supplemented with 0.2% casamino acids, MexL transcriptionally represses *mexJK* expression by approximately 4 fold. In LB however, the repressional activity was only approximately 2 fold. Similar results were obtained when measuring β -Gal activities in *E. coli* λ Mex lysogens, which contained a chromosomal *mexJ-lacZ* fusion. However, the overall β -Gal activity in the *E. coli* lysogens were much lower than those obtained in *P. aeruginosa*.

A plasmid borne *mexJ-lacZ* fusion, pPS1181, was also used to measure *mexJK* regulation by MexL. PAO238 and PAO318 harboring pPS1181 were grown in LB, and showed that MexL repressed *mexJ-lacZ* transcription approximately 15 fold. However, since pTZ110, which is the plasmid backbone of pPS1181, is a medium copy number plasmid, values obtained with pPS1181 may not accurately represent MexL repression. Chromosomal *mexJ-lacZ* fusions, therefore probably allowed a much more accurate depiction of MexL repression.

Although the conclusions of this study are relatively simple, several potential leads have been generated that may prove beneficial to future research endeavors. For example, the different β -Gal units obtained from the *P. aeruginosa* and *E. coli* fusion strains suggests that *P. aeruginosa* may express a transcriptional activator that is absent in *E. coli*. Furthermore, in both of these strains, MexL transcriptionally repressed *mexJK* expression only 2 fold when grown in LB. This finding suggests that perhaps an "inducer" of the *mexJK* efflux pump may be present in LB, and may therefore allow derepression of this pump.

Although the repressional activity of MexL obtained with *P. aeruginosa* and *E. coli* contained *mexJ-lacZ* fusions was similar, 4 fold repression in M9-casamino acid and 2 fold repression in LB, the β -Gal unit values obtained from both organisms were strikingly different. The β -Gal units obtained from λ Mex *E. coli* lysogens grown in M9-casamino acids ranged from 200 u without MexL and 50 u with MexL.¹ The β -Gal units obtained from PAO320 grown in M9-casamino acids, however were ~2600 u without MexL and ~700 u with MexL. Therefore, the following question remains to be answered: Why are the β -Gal units in *E. coli* 13-14 fold lower than the β -Gal units obtained from *P. aeruginosa*? Is there an activator present in *P. aeruginosa* that does not exist in *E. coli*, or is the promoter of *mexJK* not as efficient in *E. coli* as it is in *P. aeruginosa*?

An equally interesting finding is the repressional activity expressed when cells are grown in LB versus M9 minimal media. In both the *E. coli* and *P. aeruginosa* fusions, MexL repression was never greater than 2 fold when grown in LB. What in LB is allowing for the expression of *mexJK* despite the presence of repressor protein MexL?

Perhaps, LB contained a natural substrate(s) of the MexJK efflux pump, and therefore promoted its expression.

What is the positive regulator gene that mediates *mexL* expression? PAO238-1 expressed *mexJK* because of a point mutation in the *mexL* gene, but what activates *mexL* expression naturally? When PAO321, a *mexL* (point mutant)-*mexJ-lacZ* fusion strain, was assayed without complementation, it had a 6 fold increase in β -gal activity above PAO320, which is a *mexJ-lacZ* fusion strain. Clearly, something about the conformation of the mutated MexL from PAO238-1 activated *mexJK* expression as opposed to having the expected null effect. It may be possible that the PAO238-1 MexL assumes the same shape of a wt MexL, which would be complexed with an activator protein, and therefore not only ceases to repress *mexJK* expression, but actually enhances its expression.

The MexJK efflux pump is unique in that it lacks its own outer membrane protein channel. In fact, MexJK does not require an outer membrane protein to efflux triclosan. However, Chuanchuen et al. (12) have shown that the outer membrane protein OprM is required for antibiotic efflux. This is consistent with the MICs obtained from PAO318 (table 3.1), which did not show an increase in MICs for antibiotics.

It is currently unknown where triclosan ultimately ends up after it is effluxed. It is possible that another outer membrane protein(s) may be involved. It is also plausible that triclosan is simply translocated into the periplasm where it is chemically inactivated.

Most of the physiology of the Mex family of efflux pumps in *P. aeruginosa* has yet to be elucidated. The MexJK efflux pump system is certainly no exception. For instance, the *mexL-mexJK* intergenic region is relatively small, only 94 bp long. It is currently unknown where in the intergenic region MexL binds.

The purpose of this study was to determine the regulatory mechanism(s) of a newly characterized RND efflux pump in *P. aeruginosa*, MexJK. The data obtained from the mutagenesis and regulatory studies, established that MexL is a transcriptional regulator of the *mexJK* efflux operon.

However, the importance of MexL regulation is yet to be determined. The importance of the MexJK efflux pump in immunosuppressed and CF patients, if any, is also a mystery. Future experiments will certainly involve screening clinical isolates of *P. aeruginosa* for MexJK efflux expression.

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